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Review

Islet Identity in Transplantation Procedures: The Intersection of Cellular Maturity and Function

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Abstract

Pancreatic islet transplantation holds promise for patients with insulin-dependent diabetes, but is severely limited by a shortage of cadaveric donor islets, and more so because of stringent inclusion criteria for organ donation including donor metabolic function, age, and comorbidities. The impact of these diverse factors on islet health has led to a broad investigation of global influences on islet biology, not least of all, characterization of mature, functional cellular identity and maintenance of appropriate endocrine lineage. This review will present the current knowledge on β-cell heterogeneity and inherent plasticity, and the role of cellular dedifferentiation of islets and β-cells in the normal and pathophysiological states, including aging, diabetes subtypes, and islet transplantation. Examination of potential strategies for reduction of metabolic stress by endogenous and exogenous modes is further discussed.

Keywords

1. Summary

Over the past two decades, the Edmonton Protocol made islet allotransplantation a feasible clinical procedure which has resulted in over 1500 patients being transplanted in international centers [1, 2]. Islet transplantation is an effective and relatively noninvasive procedure for restoring endogenous insulin production and protecting against both severe hypoglycemia and microvascular complications [3-7], thus improving glycemic control and quality of life even in those grafts which retain only partial function [8, 9]. In recent years, advances in islet transplantation procedures such as standardization of islet processing, engraftment, immunosuppression/immunomodulation regimens, and identification of dynamic islet donor factors has resulted in improved clinical outcomes [10-13]. However, due to sustained cadaveric islet shortages and immunosuppression requirements, the majority of patients are not currrently eligible for transplantation, restricting this technique only to those with hypoglycemia unawareness and/or end-stage renal disease [14, 15]; moreover, those patients who do meet inclusion criteria still require 2-3 repeated transplants to achieve insulin independence, severely limiting the number of benefitting recipients. Thus, implementation of islet transplantation to treat more patients requires a deeper understanding of donor factors such as islet- and β-cell heterogeneity, and the relationship between β-cell identity and function.

2. Beta-Cell Heterogeneity: Development, Aging, and Inherent Plasticity

Pancreatic β-cell identity has been shown to be a surprisingly fluid state, changing with age, development, and disease. Although not a new concept, cellular differentiation from the mature state has gained traction as a physiological response to stress or external stimuli, in an attempt to effect the cell's survival or to re-establish homeostasis. Loss of cell identity can be defined in two broad ways: dedifferentiation, the loss of defining, functional, and/or mature cellular characteristics from a terminally differentiated cell type [16, 17]; and transdifferentiation, the change from one mature cell type to another, which can take the route of a direct transition, or alternatively, to utilize an intermediate state [16]. Transition between "mature" to "dedifferentiated" states has been shown to result from opposing influences of context-dependent stress evasion/survival and regeneration forces. Importantly, the ability to direct this process is of critical importance for the treatment of diabetes.

While generation of functional β-cell mass from non- β-cell-source is of critical interest and hope for diabetes therapy, this review will focus on β-cell heterogeneity *in vivo* specifically; in the least, utilization of induced pluripotent stem cells (iPSc) and human embryonic stem cell (HESc) source for organ renewal is both optimistic and provocative in its scope. Elegant reviews of the endocrine pancreas developmental program [18, 19], and novel protocols for the attempt at recapitulation and exploitation of these processes *in vitro* and *in vivo* [20-28] for functional islet mass generation provide a framework for the current examination of islet cell identity in the transplantation milieu.

Insulin hormone presence is the standard-bearer for β-cell identity, however other components must be present and functional, which include glucose sensing, cell excitability, insulin processing

and secretion, and the presence of specific transcription factors (TFs) which control the transcriptional programs that provide for the other endocrine functional traits listed [29]. Under normal circumstances, β-cell replication in the mature mammalian pancreas is extremely low [30, 31], although compensatory increases in proliferation have been documented, such as obesity, high-fat diets, injury models, and pregnancy [32-35]. Evidence for the loss of β-cell proliferative capacity linked to aging and T2D have been proposed [36-38], and may be mediated by p16[ink4a] [39]. Indeed, β-cell proliferation has been shown to be a state of phenotypic immaturity, necessary for cellular growth and modulated by the master regulator gene c-Myc [40]. Others have further suggested that transient β-cell dedifferentiation during proliferation involves a transition to a PP-positive cell state, and under the control of Smad7 [41].

Multiple examples of *in vivo* β-cell lineage plasticity have been described. In both mice and human pancreas, a subpopulation of rare lineage-plastic β-cells was identified, generating progeny with non- β (α, δ, γ), pancreatic ductal, and even neural cell fates [42-44]. These cells were broadly classified by their increased proliferative capacity, and a lack of functional and maturation markers, including the glucose transporter-2 Glut2 (SLC2A2) [42, 45]. Intriguingly, these cells maintain insulin expression, albeit in lower levels than in fully functional endocrine cells [42]. In the early postnatal mouse and human pancreas, these cells were found in higher proportion in the small, extra-islet clusters of β-cells than in prototypical islets [43]; as the endocrine pancreas remodels with age, these cells decrease in proportion [46], paralleling the decrease in β-cell proliferative capacity and the simultaneous increase in functional glucose responsiveness. Importantly, these cells proliferate during times of increased metabolic demand, such as diabetes [47] and following exposure to the specific β-cell toxin, streptozotocin [48], thus attempting to replenish functional β-cell mass and maintenance of glucose homeostasis. Moreover, these cells show a robust proportional increase in the pregnant female mouse -- a classic model of rare postnatal β-cell proliferation -- immediately preceding the increase in β-cell mass required for fetal somatic growth and in response to hormonal influence [48]. These Ins$^+$Glut2^{LO} cells subsequently decrease shortly before parturition, allowing appropriate endocrine pancreas remodeling in the peri-gestational and postpartum period [49].

The genetic signature of fate-undefined β-cells has been rigorously examined; in addition to low insulin and a relative lack of Glut2, these Ins$^+$Glut2^{LO} cells demonstrate alterations in the relative expression levels of MafB, Pdx1, Nkx6.1, and Ngn3 [42, 43], suggesting an immature β-cell phenotype *in vivo*. Others have similarly showed a population of Pdx1$^+$Ins^{low} β-cells which demonstrates low expression of Glut2, Gck, and MafA, whilst being proliferative [50]. In yet another example, a collection of insulin-lineage reporter-labelled β-cells was found to demonstrate an immature phenotype, with increased expression of Pdx1 and MafB, and a lack of Nkx6.1 and Glut2 [51]. In these and other examples, the cells of interest participated in a renewal of β-cell mass during aging and after injury [50-52].

This immature β-cell phenotypic signature has been further reinforced, such as the lack of the β-cell maturation marker Urocortin 3 (Ucn) [53, 54]. The spatial orientation of these Ucn3-deficient β-cells was suggested to be at the islet periphery, in what the authors' term a "neogenic niche" for "virgin" β-cells [55]. These cells were proposed to be the result of early transdifferentiation from α-cells, representing an early intermediate stage of development, but which persist throughout life, decreasing in proportion with age [55]. Bader and colleagues have shown that *Flattop* (*Fltp*), a planar-cell polarity gene responsible for maintenance of the rosette

structure of cells within the pancreas can be used to distinguish mature from proliferative β-cells [56]. The *Fltp*-negative, proliferative β-cells contained less Glut2, Ucn3, MafA, and Nkx6.1, which echoed findings from others [42, 43, 53, 57]. In another study, Johnston et al reported that β-cell "hubs" exist, intermingled amongst other β-cells within the islet; analogous to a pacemaker, these specialized β-cells dictate responses to glucose [58]. These hub β-cells represent less than 10% of cells by total number, demonstrate a transcriptionally immature phenotype, and are highly metabolically active. And in yet a further example, Westacott and others found that these pacemaker β-cells control electrical dynamics, thus controlling insulin secretion [59]. These data suggest that islet architecture, including size or number of cells within an islet, location of β-cells within the islet or pancreas, and planar orientation, has direct impact on islet function. Whether these cells represent a precursor/resident stem cell population, or simply a population of β-cells which can be recruited in times of increased metabolic demand, may be a qualified argument, as many of the same markers define early and late (or, alternatively, mature and immature) β-cells, albeit in varying amounts. However, their utility in tissue repair remains steadfast, and further information is required to identify, and/or protect them clinically, particularly when making islet transplantation decisions. This further provides enhanced rationale for protecting existing cell populations, as these data deliver convincing evidence that islet cells do not exclusively die during disease progression, as was the canonical assumption; instead, a population of them survive, but cease to behave as functional endocrine cells.

These data also show the prevalence and breadth of β-cell heterogeneity. Beta-cell phenotypic non-conformity was quantified by mass cytometry in isolated human islets using surface markers for various hormones and proliferation. Provocative results from "barcoding" cell populations suggest that β-cells exist within many cell states, specifically within those proliferating endocrine cells [60]. Three major clusters of β-cells within individuals were reported, correlating roughly with proliferation and age metrics [60]. A similar study found that β-cells occupied one of four states when assessed by the differential expression of ST8SIA1 and CD9 [61]. The majority of β-cells were absent for both ST8SIA1 and CD9 in healthy donors; in contrast, ST8SIA1 presence was markedly increased in islets from donors with T2DM. The ST8SIA1-positive β-cells were less glucose responsive, suggesting functional and clinical deficits [61]. In yet another study, isolated single β-cells were shown to exist within at least 2 states by software analysis of Ca^{2+} responses to high glucose [62]. Unsurprisingly, there is also evidence for sex differences underlying β-cell function and pathology [63], including GSIS assessments and DNA methylation patterns [64]. Importantly, these changes are not only found to be variable between pancreata of different donors, but also within the pancreas of the same donor, layering complexity onto our understanding of islet biology.

3. The Genetic Makeup of β-Cells Defines Their Function

The presence or absence of specific genes, transcription factors, and proteins has been proposed to maintain or change β-cell fate. Puri *et al* showed that deletion of the von Hippel-Lindau (*Vhl*) gene, which regulates the hypoxic response via the transcription factor hypoxia-inducible factor (HIF), causes negative effects to β-cell gene expression. They and others have found a relationship between the HIF-hypoxia response pathway and glucose homeostasis: the loss of *Vhl* effected a phenotype similar to diabetes and caused alterations to endocrine identity

[65, 66]. The authors attributed this change to the inappropriate maintenance of Sox9, present in β-cell progenitors but normally excluded from mature β-cells. Thus, under hypoxic conditions, the β-cell dedifferentiates in order to survive.

Nkx2.2 is expressed in the developing pancreas and later restricted to the endocrine-lineage, including α, β, PP, and ε-cells [67]. In the adult, Nkx2.2 is maintained in α, β, and PP-cells. Genetic deletion of Nkx2.2 was shown to decrease generation of α and PP cells, and halt β-cell specification, causing lethal hyperglycemia. These lineage-unspecified cells produced none of the four endocrine hormones, but did express some β-cell markers including Pdx1 and IAPP, the protein component of islet amyloid, islet amyloid polypeptide (IAPP, or amylin), yet lacked Glut2 and Nkx6.1 [68]. Nkx2.2 is part of a large repression complex in β-cells that includes DNMT3a, Grg3, and HDAC1, and prevents β- to α- cell reprogramming [69]. Guttierez *et al* recently demonstrated that deletion of Nkx2.2 in mature mouse β-cells caused rapid onset of hyperglycemia, loss of insulin production, and down-regulation of β-cell genes. Furthermore, there was evidence of cellular reprogramming and bihormonal cells in the Nkx2.2-null β-cells that acquired non–β cell endocrine features, suggesting that β-cell identity requires continual repression of non–β cell programs [70].

The TF Arx normally specifies α-cell fate and simultaneously represses the β- and δ- lineages [71]. In a gain-of-function experiment, forced expression of Arx in the embryonic pancreas or in developing islet cells caused fatally high hyperglycemia in mice. This was a result of near-complete loss of β- and δ- cells, and a concurrent increase in the proportion of α- and PP- cells. Intriguingly, the mis-expression of Arx in adult β-cells *in vivo* resulted in their conversion to α- and PP- cells [72]. Alpha-to-beta transdifferentiation and α-cell identity loss has been effected by targeting Arx, shown in multiple reports [73, 74], and in one report, with concurrent targeting of DNA methyltransferase 1 (Dnmt1) [75]. Interestingly, Wilcox et al reported that the Arx-negative cells which underwent a transdifferentiation via a bihormonal state expressed β-cell markers including Pdx1, MafA, and Glut2 in the neonate, but not adult mouse β-cells [74]. Li et al reported that artemisinins, antimalarial drugs that repress Arx by causing its translocation to the cytoplasm, could be used as a cell source for β-cell replacement strategies for T1DM patients. The authors purported that artemisinins, including artemether, increase GABA signaling and prevent glucagon secretion by α-cells, resulting in their consequent loss of identity and transdifferentiation to a β-like phenotype, with associated increased insulin content and improvement in GSIS assessments [76]. However, follow up reports challenged these results, showing that neither artemisinins nor GABA directly cause α- to β-cell conversion [77, 78].

The TF Pax4 has been shown to specify β-cells from endocrine progenitors [79]. In an experiment of ectopic Pax4 expression in both endocrine progenitor and adult endocrine cells, α-cells were re-specified towards a β-cell phenotype, generating oversized islets [80]. A subsequent model utilizing double-transgenic mice of inducible, ectopic, and reversible expression of Pax4 in glucagon-positive cells showed that adult α-cells can be converted to β-cells [81]. The authors report this to be Ngn3-dependent which redirects precursor cells in the duct to develop as β-cells via an α-cell intermediary state, and capable of β-cell mass replenishment after targeted depletion [81]. Pax6 is similarly necessary to maintain β-cell identity, as deletion in the adult causes severe hyperglycemia, reduced GSIS, deficient glucose-sensing, and down-regulation of key β-cell genes; this deletion also "un-inhibited" selectively repressed genes for the β-cell program [82].

Injury models have demonstrated the attempts of the pancreas to replace lost β-cell mass, sometimes in unorthodox ways. Alpha cells were shown to transdifferentiate to β-cells after extreme loss [83], and after forced expression of Pdx1 [84]. It was later shown that Pdx1 maintains β-cell identity by repressing the α-cell program [85]. Chera *et al* reported that the cell type transdifferentiating changes with age in RIP-DTR mice, whose β-cells bear DT receptors: after >99% β-cell loss due to diphtheria toxin exposure in young animals, the β-cell compartment was reconstituted by transdifferentiation from δ-cells; after puberty, the α-cells were instead the regenerating cell type [86].

Islet environmental influences also impact heterogeneity and identity. Using proteomics, Mahmoud *et al* classified factors for the maintenance of β-cell stability and function during culture procedures, particularly in the interim between islet donor procurement and recipient transplant infusion [87]. Short and long-term *in vitro* conditions have been shown to induce a loss of mature islet-cell phenotype, including gene expression and GSIS metrics. The authors found that an extracellular protein, Apolipoprotein E (ApoE), significantly increased the expression of key β-cell genes Ins2, Nkx2.2, Nkx6.1, Ucn3 and Glut2, which had been down-regulated after 4 weeks in low glucose [88]. Furthermore, Spijker et al demonstrated that dispersed human β-cells can undergo a conversion into glucagon-producing α-cells which are indistinguishable from innate α-cells [89]. These cells also retain their α-cell phenotype after transplantation in vivo. This fate conversion occurs after β-cell degranulation, and is characterized by Pdx1 and Nkx6.1 presence in glucagon^{+} cells. Interestingly, the transdifferentiation process can be blocked by lentiviral inhibition of Arx, further supporting the importance of this TF for α-lineage specification [89].

While variable in their experimental methodologies, rationales, and results, the data shown here demonstrate substantial evidence supporting the existence and variability in β-cell heterogeneity, and how loss or gain of specific genes has substantial effect on cell function.

4. Beta Cell Dedifferentiation as A Result of Hyperglycemia and Diabetes Mellitus

Examples of β-cell dysfunction and consequent dedifferentiation caused by metabolic stress have emerged as a critically topical explanation for the epidemic of patients with T2DM; the most recent data available from 2017 shows that 9.7% of the US adult general population has diabetes, 91.2% of those with T2DM [90]. This number does not include those with pre-diabetes, impaired fasting glucose, nor patients with metabolic syndrome, thus estimation of the global impact of impaired β-cell function is widely underestimated.

Many describe the islet stress process as metabolic exhaustion from chronic load, leading to severe β-cell dysfunction [91]. Multiple mechanisms have been attributed, including oxidative stress from glucose metabolism [92, 93], and endoplasmic reticulum (ER) strain, specifically the unfolded protein response (UPR), causing impaired insulin secretion [94-96]. Lu *et al* depicts β-cell loss of identity as the result of a two-hit hypothesis: sustained metabolic stress on the β-cell, and loss of polycomb-silencing via PRC2, triggering dedifferentiation of β-cells and consequent diabetes [97]. Polycomb comprises two repressive complexes, PRC1 and PRC2, which control gene silencing [98]. It is likely that a combination of many factors contribute to islet loss-of-function.

Alleviation of metabolic over-demand on the β-cell is an obvious first step. From the perspective of the islet, attempts to promote β-cell "rest" is not new, such as exogenous insulin supplementation, which turns off endogenous production [99]. New techniques now exist to

achieve this metabolic stasis, including lowering the glucose set-point, improving insulin sensitivity following treatment with thiazolidinedione (TZD) therapy, glucagon-like-peptide-1 receptor (GLP-1R) agonism alone, and/or in combination with sodium/glucose cotransporter-2 inhibition (SGLT-2i), which have proven effective in a mouse model of T2DM using β-cell-deficient obese hyperglycemic/hyperinsulinemic KS *db/db* mice [100].

Beta-cell dedifferentiation and functional changes with insulin resistance (IR) in the absence of hyperglycemia have been documented, which illustrate the natural history of disease progression. Aguayo-Mazzucato reported that mouse β-cell aging markers have heterogeneous distribution and are induced by IR; indeed, these cells can be stratified by the relative presence of the insulin-like growth factor 1 receptor (IGF1R) and aging markers p16^{ink4a}, p53BP1, and senescence-associated β-galactosidase. Importantly, these aging markers increased after the introduction of insulin resistance or ER stress [101]. Fiori *et al* examined non-human primates fed a high-fat/high-sugar "westernized" diet (HFS) for 24 months, with and without the polyphenol resveratrol [102] (HFS+Resv), compared to a standard-diet (SD) group. Increased GSIS and IR were documented in both HFS and HFS+Resv diets compared with SD [103]. There were alterations in α- and β-cell proportions, and an increase in α-cells containing glucagon and GLP-1 with HFS diet suggesting β-cell dedifferentiation, whereas HFS+Resv islets were morphologically similar to SD. And comparable to other studies recorded, HFS islets demonstrated a decrease in mature β-cell transcription factors including FoxO1, Nkx6.1, Nkx2.2, and Pdx1, which did not occur with resveratrol supplementation. Similar changes were observed in human islets *in vitro*, and suggest that the effects of resveratrol are mediated through Sirtuin 1 (Sirt1) [104, 105], as the islets showed a significantly decreased insulin secretion response when a Sirt1 inhibitor was applied [103]. Islet morphology was further shown to be altered in non-diabetic, insulin-resistant human islets relative to insulin-sensitive islets, demonstrating increased islet size and an elevated number of β- and α-cells that resulted in an altered β-cell to α-cell area in the IR group [106].

As IR and impaired glycemia progress to overt diabetes, the loss of mature β- and α-cell phenotypes similarly manifest. Hyperglycemia was shown to reversibly alter islet structure and function, such that β-cell-specific expression of a human activating K_{ATP} channel mutation in adult mice led to rapid diabetes and defined alterations in islet morphology, ultrastructure, and gene expression [107]. Sustained hyperglycemia was shown to be associated with a reduction in insulin-positive cells and an increase in glucagon-positive cells in islets, without alterations in cell turnover. Furthermore, some β-cells began expressing glucagon, whilst retaining many β-cell characteristics, representing identity loss. Hyperglycemia, rather than K_{ATP} channel activation, underlies these changes, as they were prevented by insulin therapy and reversed by sulphonylureas [107]. Talchai and colleagues described β-cell dedifferentiation as a mechanism of β-cell failure in type 2 diabetes. Using a mouse model of FoxO1-deficient β-cells, the authors reported that FoxO1 ablation caused hyperglycemia with reduced β-cell mass following canonical drivers of metabolic stress, such as pregnancy and aging [108]. The loss of β-cell mass was shown to be the result of dedifferentiation and not β-cell death, challenging established paradigms [109]. Furthermore, these dedifferentiated β-cells reverted to progenitor-like phenotypes, expressing Ngn3, Oct4, Nanog, and L-Myc. A minority of FoxO1-deficient β-cells adopted the α-cell phenotype, expressing glucagon. These data demonstrate that FoxO1 is required to maintain the β-cell fate under metabolic stress, and Pdx1, MafA, and Nkx6.1 alone are insufficient to maintain β-cell fate in the hyperglycemic metabolic environment [108]. Mis-expression of similar transcription factors

present in islets from donors with T2DM has been further demonstrated, shown by altered MafA and Pax4 presence in α-cells [110]. In another example from the Accili group, human β-cell dedifferentiation was scored using multiple indicators of endocrine lineage (hormones), markers of mature endocrine function (synaptophysin and chromogranin A), transcription factors, and an endocrine progenitor cell marker, aldehyde dehydrogenase 1A3 (ALDH1A3). Comparing pancreata from control non-diabetic (ND) *vs.* T2DM donors, dedifferentiated cells represented 31.9% of β-cells in T2DM *vs.* 8.7% in ND, and 16.8% *vs.* 6.5% of all endocrine cells. The number of ALDH1A3-positive/hormone-negative cells was shown to be 3-fold higher in T2DM than ND controls. There was further evidence of β-cell specific transcription factors present in glucagon- and somatostatin-expressing cells of donor samples with diabetes [111]. In another study, Spijker *et al* demonstrated an 8-fold increase in bihormonal, insulin$^+$glucagon$^+$ cell presence in T2DM *vs.* ND human islets (4.05% vs 0.52%), affecting 33% of the islets in pancreata with T2DM *vs.* 13% in ND control donors [112]. These bihormonal cells demonstrated an exclusively non-nuclear MafA expression pattern, and FoxO1 exhibited disparity in compartment staining presence, being found primarily in the cytoplasm of ND-control islets, as compared to the nucleus of T2DM cells. Furthermore, nuclear Pdx1 was expressed in a subset of glucagon$^+$Nkx6.1$^+$ cells; overall, these results suggest diabetes-driven endocrine-cell dedifferentiation [112]. Furthermore, there was a correlation between the bihormonal cells and Nkx6.1$^+$glucagon$^+$insulin$^-$ cells from samples with diabetes, and the presence and extent of islet amyloidosis [112]. IAPP is produced by the β-cell and co-released with insulin in response to stimuli; IAPP is normally soluble in its monomeric state, but forms misfolded islet amyloid in type 2 diabetes [113]. Further evidence from Bader et al showed that the human *Fltp* ortholog Cfap126, as well as SLC2A2 and Pdx1, were significantly down-regulated in human islets from pre-diabetic donors, and further down-regulated in islets from T2DM donors compared to ND samples, suggesting that Wnt/PCP signaling and β-cell maturation and/or function are impaired by hyperglycemia and altered metabolic status [56]. The β-cell maturation marker Ucn3 was further shown to be co-secreted with insulin upon glucose sensing, promoting somatostatin release from δ-cells, ensuring appropriate responses to glucose fluctuation in a feedback loop with insulin and glucagon. Importantly, Ucn3 was shown to be depleted from β-cells in multiple examples of diabetes and sustained hyperglycemia, specifically in humans with T2DM [114]. Rescue of dedifferentiated Ucn3-negative β-cells was reported by the addition of a small molecule inhibitor of TGFβ receptor I, Alk5 inhibitor II. It was found to restore mature pancreatic β-cell identity after extended hyperglycemia and exposure to inflammatory cytokines, re-expressing Ucn3 and key β-cell transcription factors [115]. In yet another paper, Dahan *et al* showed that a subset of β- and δ- cells in islets from donors with T2DM expressed the stomach hormone gastrin, which is otherwise expressed in the pancreas only during embryogenesis [116]. *In vivo* and *in vitro* data demonstrate that gastrin expression in the β-cell was eradicated after restitution of normoglycemia [116]. And, in recently diagnosed pancreas samples with T2DM, β-cells showed evidence of α-cell colocalization, as well as the presence of the mesenchymal marker vimentin, indicating both β-cell dedifferentiation and reprogramming to other cell lineages [117]. The presence of vimentin in the endocrine cells of patients with T2DM has been similarly shown by others, demonstrating a higher expression in α-cells than β-cells, and relative to ND-control donor samples [118]. These vimentin-positive β-cells were not apoptotic, and had reduced expression of Nkx6.1 and Pdx1. However, these dual-lineage cells were neither associated with islet amyloidosis,

nor with bihormonal expression (insulin + glucagon) [118], contrary to other groups' findings described above [104, 107, 108, 113].

There is also emerging evidence for β-cell dedifferentiation in T1DM. Examination of remnant β-cells and isolated islets from T1DM donors illustrated retained insulin secretion regulation, although there was a reduction in MafA and Nkx2.2 gene expression; α-cells however demonstrated loss of function, as well as variation in α- and β-cell lineage programs [119]. Implantation of islets into an immune-protected *in vivo* environment allowed for partial recovery of α-cell phenotypic identity, but no change in β-cell function, suggesting that α-cell dysregulation contributes to disease burden in addition to established β-cell loss [119]. Lam *et al* examined a large cohort of human pancreas samples (+/- T1DM) for assessment of β-cell mass, endocrine cell turnover by proliferation and apoptosis, and co-expression of insulin and glucagon. The authors demonstrated a minimal proportion of transdifferentiated or bihormonal cell presence; only 0.01% Gcg+ARX+Nkx6.1+, 0.02% Gcg+ARX+Nkx6.1+, and 0% Gcg+ARX+Ins+Nkx6.1+ cells were detected in ND pancreata. T1DM pancreata, in comparison, did not contain any cells expressing all 4 of the α- and β-cell markers tested. The authors thus concluded that there was no apparent, or measurable, transdifferentiation using these techniques [120]. However, these conditions may be too stringent, as multiple other groups have shown loss of the mature markers Arx and Nkx6.1 in dedifferentiated cells, and Nkx6.1 is required for mature β-cell function, a state incongruent with dedifferentiation [121]. Interestingly however, a recent report from the same group demonstrated a highly proliferative population of cells present in adolescent and young adult donors with and without T1DM. These cells expressed Arx and cytoplasmic Sox9; furthermore, about 1/3 expressed glucagon, although the majority were hormone-negative [122]. These data suggest lineage plasticity within adolescent and adult human pancreata, and further implicate the role of the α-cell. As T1DM is an autoimmune disease, one group identified a subset of immune-resistant β-cells in a mouse non-obese-diabetic (NOD) model. Rui and others suggestedthat this sub-population develops from normal β-cells after immune attack, and demonstrated reduced expression of genes associated with mature β-cell identity [123].The authors provide evidence for a cell survival program activation; alternatively this could be a consequence of dedifferentiation in response to autoimmunity.

5. Endocrine Cell Dedifferentiation in the Transplantation Setting

As evidenced above, islet phenotype can be altered by its environment. Due to inflammation and exocrine pancreas insufficiency consequent to chronic pancreatitis, extensive damage to the endocrine compartment can result in pancreatogenic or type 3c diabetes [124]. Similarly, cystic fibrosis–related diabetes (CFRD) is a common and severe complication of CF, which can consequently require islet allo-transplantation. Recently, Hart *et al* showed that ~65% of β-cell area was lost in CF pancreata, evidenced by pancreatic remodeling, inflammation, and peri-islet adipocyte and fibrosis deposition, and not by inherent islet abnormalities in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The authors further found that the islet niche and islet morphology were altered in CF, which likely impacts insulin and glucagon secretion [125].

Chiefly in the transplant setting, cell identity maintenance is of critical importance. However, there is relative scarcity of data available. Anderson *et al* examined islet cell identity in two retrieved intraportal islet allotransplant grafts from deceased recipients who maintained excellent

graft function and continuous insulin independence for 13 and 3 years. The authors describe a loss of Ucn3 presence as well as glucagon colocalization in insulin-expressing β-cells present; in contrast, no Ucn3-negative β-cells were seen in non-diabetic deceased donor control islets in pancreas sections [126]. The authors postulated this to be a dedifferentiation survival mechanism to counteract the stress caused by islet isolation, including ischemia and consequent changes to vasculature during engraftment [126]. While there is no way to assess the timing of the islet dedifferentiation retrospectively (i.e., pre – or post- transplant), and the small sample size studied precludes broad conclusions, these data suggest that loss of mature cell phenotype is a persistent issue warranting further investigation. In a separate report, the same patient sample whose transplant functioned for 13 years showed little-to-no amyloid deposition in the liver, suggesting retained function and low cytotoxic effects [127]. Islet amyloid has been shown to lead to progressive β-cell dysfunction and cell death, contributing to islet transplantation failure [113].

Mezza *et al* examined changes in islet morphology secondary to insulin resistance from pancreas samples obtained during 50% pancreato-duodenectomy surgery, from non-diabetic subjects classified as insulin-resistant or insulin-sensitive. In addition to changes in insulin secretion and sensitivity assessments, and alterations in α- and β- size and proportion, the authors found that 77.7% of patients who were insulin resistant before surgery subsequently developed type 2 diabetes thereafter [106].

Recently, we sought to investigate the identity of islets in chronic pancreatitis patients undergoing pancreatectomy and islet auto-transplantation (PIAT), and if long-term transplant success could be compared with β-cell functional phenotype status at the time of transplant. All patients were non-diabetic at the time of transplant, and all received equivalent numbers of islets (~5000 IE/kg). Using histological markers of β-cell identity including hormones, vimentin, synaptophysin, Ucn3, and MafA, we found that β-cell dedifferentiation was evident in all patients undergoing PIAT, which paralleled disease severity, and could be stratified relative to ND control and T2DM control donor samples [128]. These findings were further supported by pre- and post-surgical metabolic function tests, HbA_{1C}, and GSIS assessments with up to 4 years follow-up. Results indicated that pre-surgical insulin resistance was associated with loss of β-cell phenotype, and hence function, at the time of surgery, and might suggest a possible pre-surgical tool which can inform transplant success [128]. These data further indicate that islet cell quality and maturity is paramount for long-term glucose homeostasis following transplantation. Furthermore, disease duration and degree of damage may impact the quality of cells available for transplant. Thus, islet identity may be impacted before and after transplantation, and should be considered when making clinical decisions including donor selection criteria.

6. Targeting the Reduction of Metabolic Stress: The Bone-Pancreas Connection

Multiple questions arise from these data, such as (how) can we identify islet or β-cell heterogeneity with diagnostic testing in living humans; if so, can we apply a standard metric to a threshold value; and related, is there an optimal time for intervention? Furthermore, is there a way to maintain or promote a mature and functional β-cell identity and phenotype in the global rise of insulin resistance, metabolic syndrome, and type 2 diabetes?

Clinical strategies for reversal of damage caused by hyperglycemia, pancreatic disease, and metabolic syndrome will be of critical importance moving forward. One potential target is

osteocalcin (OC), an osteoblast-derived hormone found to have global body effects. The link between bone health, energy metabolism, and glucose regulation has been known for some time [129-132], although the mechanism of action only now is being elucidated [133, 134]. Osteocalcin-deficient mice are mildly hyperglycemic [132]; insulin receptors are present on osteoblasts, and insulin signaling here regulates body composition [135]; and the ligand for osteocalcin, the G-protein coupled receptor-6a (GPRC6a), is found on multiple cell types, including β-cells, allowing the undercarboxylated form of osteocalcin (u-OC) [136] to bind in a bone-pancreas feed-forward loop [137]. This signaling axis was supported by the finding that global GPRC6a$^{-/-}$ mice had both osteopenia and metabolic syndrome, with glucose intolerance and fatty liver [138]. Osteocalcin has been found to contribute to the regulation of glucose tolerance, and insulin secretion and sensitivity in experimental animals, and serum osteocalcin concentration was inversely associated with blood markers of metabolic syndrome [139]. Importantly, this showed that disease progress could be quantified by the relative presence of osteocalcin, which putatively could be used as a diagnostic tool. Osteocalcin was further shown to induce β-cell proliferation by binding to GPRC6a; inactivation of this receptor in β-cells caused a reduction of insulin production and glucose intolerance. Importantly, this regulation occurs during both development and in adulthood [140]. In mice with β-cell-specific *GPRC6a* deletion, there were reductions in pancreas weight, islet number, insulin content, and insulin gene expression. Both islet size and β-cell proliferation were reduced in *Gprc6a*$^{β-cell-cko}$ mice compared with control mice, and the knock-out animals exhibited altered glucose tolerance, yet normal insulin sensitivity [141]. Furthermore, isolated islets from the β-specific GPCR6a-deficient mice demonstrated reduced GSIS in response to osteocalcin. Thus, the authors provide evidence that GPRC6a is a receptor for OC, and present a direct role for GPRC6a in regulating β-cell functions, including mass and insulin secretion [141]. Osteocalcin treatment of islets was further shown to increase both Ins1 and Ins2 insulin genes as well as cyclin-dependent kinases and cyclin D1 and D2, promoting β-cell proliferation [133, 142].

Our laboratory has shown that osteocalcin protects against nonalcoholic steatohepatitis (NASH) in a diet-induced mouse model of metabolic syndrome, targeting inflammation and fibrosis in liver and white adipose tissue [143]. In this model using middle-aged *Ldlr*$^{-/-}$ mice fed a Western-style, high fat/ high cholesterol diet, uOC treatment furthermore protected against insulin resistance [143]. We subsequently undertook an investigation of osteocalcin on isolated human islets from 22 donors, and showed that uOC-supplementation *in vitro* increased multiple targets, including insulin content, the proportion of β-cells relative to α-cells, islet-cell proliferation, and the expression of the sulphonylurea receptor Sur1 [144]. Sur1 is important for glucose homeostasis and insulin secretion [145]. After transplantation into Nod.scid mice for 1 month, human islet grafts supplemented with 4.5 ng/ml decarboxylated-OC demonstrated increased human insulin and C-peptide content, enhanced *in vivo* GSIS response, and decreased presence of cells bihormonal for insulin and glucagon relative to non-supplemented islets, suggesting that osteocalcin contributed to endocrine cell redifferentiation [144]. Ferron et al further demonstrated that intermittent injection of osteocalcin improved glucose metabolism in mice and mitigated T2DM [146].

Subsequent evaluation of *in vivo* osteocalcin levels in children recently diagnosed with both T1DM and T2DM demonstrated an inverse correlation between HbA$_{1c}$ and serum concentrations of uncarboxylated osteocalcin, specifically that low uOC was found in patients with poor glycemic control shown by high HbA$_{1c}$ [147]. This was not significantly related to age, sex, race/ethnicity,

diabetes subtype, or body weight status, although the association between uOC and HbA$_{1c}$ was more pronounced in children with T2DM than with T1DM, and in males than females. This observation supports the known effect of OC on insulin secretion and sensitivity, which are both impaired in T2DM [147]. Alterations in uOC levels has been shown in multiple subsequent reports, including in chronic pancreatitis patients [148], in postmenopausal women with metabolic syndrome [149], and in a pediatric population with metabolic syndrome, insulin resistance and obesity [150]. Furthermore, there was lower bone turnover, and hence lower serum uOC, in Mexican American men, but not women, with T2DM who had poorer glycemic control [151]. Importantly, Sanchez-Enriquez *et al* demonstrated a link between cardiovascular risk factors for patients with T2DM, including high BMI, high fasting plasma glucose, and high HDL, and low serum levels of uOC, suggesting that osteocalcin could have more physiological roles than previously expected [152]. In a different model, patients who underwent liver transplantation and subsequently became diabetic post-surgery demonstrated that total osteocalcin was, yet again, inversely associated with plasma glucose levels and insulin resistance [153].

The finding of an inverse correlation of uOC with glycemic control warrants further hypothesis-driven research, particularly with respect to endogenous *vs.* exogenous reliance of insulin, the confounding influence of exercise, and variability in both patients and islet donors. Rigorous examination of the mechanisms responsible for osteocalcin action are underway, as well as further elucidation of the relationship between bone and endocrine health as it relates to disease mitigation. While osteocalcin as a direct therapeutic agent has not yet been shown in human trials, these data suggest its potential future utility as hormonal replacement strategy for improving glycemia in patients with metabolic syndrome or T2DM, or indeed, for increasing islet mass or function for transplants. These data underscore the importance of β-cell mature function, identity, and lineage maintenance in relation to endocrine pathologies in the clinical setting, including transplantation procedures.

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Competing Interests

The authors have declared that no competing interests exist.

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