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The Presence, Origins and Potential Role of Bi-hormonal Endocrine Cells Within the Pancreatic Islets of Langerhans

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Abstract

The pancreatic islets of Langerhans contain a minority of endocrine cells that contain more than one hormone: predominantly combinations of glucagon, insulin and somatostatin. A recent paper from our laboratory examined the ontogeny of such cells in the human pancreas and found that they persisted throughout the lifespan but altered in relative abundance with age. Glucagon/insulin bi-hormonal cell number significantly increased with age whilst insulin/somatostatin and glucagon/somatostatin cells significantly decreased. Building on that study here we explore the possible origins and physiological role of bi-hormonal cells within the endocrine pancreas. During pancreas development *in utero* mono-hormonal endocrine cell lineages are defined by distinct signatures of transcription factor expression. Insufficient or inappropriately timed expression in sub-populations of endocrine cell progenitors may fail to suppress genes normally restricted to other endocrine cell types, resulting in residual populations of bi-hormonal cells. These have been identified postnatally by single cell transcriptomic or proteomic analysis and do not appear to have a metabolic role distinct from that of mono-hormonal cells. However, during hyperglycemic stress bi-hormonal cell sub-populations can proliferate and transdifferentiate into new beta-cells capable of glucose-stimulated insulin release. Transdifferentiation is reversible and in both type 1 and 2 diabetes some beta-cells can dedifferentiate to form hormone-null cells or alpha-cells. Thus, current evidence suggests that diverse phenotypic pools of endocrine cell phenotypes are retained within the islets into adult life that can undergo lineage changes in response to metabolic demand. There is preliminary evidence that these pathways can be therapeutically manipulated.

Introduction

The pancreatic islets of Langerhans contain distinct endocrine hormone populations of which the major phenotypes are insulin-secreting beta-cells, glucagon-secreting alpha-cells, and somatostatin-secreting delta-cells [1]. Within the human pancreas these are present in the approximate ratio of 60:30:10% respectively. Other minority endocrine cell types are also present contributing 1-2% of the islets including pancreatic polypeptide secreting cells and ghrelin-secreting epsilon cells. However, a minority of each endocrine cell type appear to be bi-hormonal in nature. We recently published an analysis of the presence and ontogeny of bi-hormonal islet cells within the human pancreas across the lifespan in individuals without diabetes utilizing immunofluorescence co-localization [2]. Bi-hormonal cells were observed at all ages from birth until the eighth decade with the most abundant cell phenotype containing both glucagon and insulin, representing 3% of all islet cells. Glucagon/somatostatin, and insulin/somatostatin bi-hormonal cells were also observed but at lower abundance. We found that glucagon/insulin bi-hormonal cell number significantly increased with age whilst insulin/somatostatin and glucagon/somatostatin cells significantly decreased. While the study showed that bi-hormonal cells are present within human islets throughout life it did not shed light on the origins or physiological potential of these cell populations, or how they might change during metabolic stress or diabetes. This commentary offers some perspectives.

Bi-hormonal Cell Presence in the Healthy Pancreas

Studies by Kang *et al.* [3], used both single cell RNA sequencing (scRNA-seq) and single cell nucleus RNA sequencing to identify five sub-clusters of glucagon-expressing cells within isolated islets from healthy human donors. One cluster co-expressed glucagon/insulin and could differentiate into either glucagon or insulin mono-hormonal cells *in vitro*. When maintained as islet grafts *in vivo* though, these cells showed unidirectional plasticity to become beta-cells only. A specific gene expression signature was identified during the alpha- to beta-cell transdifferentiation including genes regulating calcium-channel function, long non-coding RNAs, DNA-binding proteins, and histone deacetylases; the latter suggesting epigenetic modification of histone structure to support the repression of genes typical of alpha-cells, such as the processing enzymes regulating the relative synthesis of glucagon versus glucagon-related peptide-1 (GLP-1), and the up-regulation of genes required for glucose-stimulated insulin secretion (GSIS).

Both Saikia *et al.* [4] and Elgamal *et al.* [5], identified distinct alpha-cell sub-clusters expressing both insulin and glucagon within human islets. When scRNA-seq was used to document the transcriptome of human pancreatic slices from non-diabetic subjects, multiple sub-clusters of insulin-expressing cells were found, with one of these co-expressing glucagon and TMSB10, a gene associated with cytoskeletal reorganization and cell motility [6]. If these cells contribute towards alpha- to beta-cell transdifferentiation this might suggest an associated anatomical restructuring of islet cell connectivity within the islets. Yu *et al.* [7] used co-expression of specific islet endocrine lineage markers in normal mouse pancreas to study the transcriptomes of rare bi-hormonal cell populations. The most frequent of these were glucagon/insulin and glucagon/pancreatic polypeptide (PPY) phenotypes, both of which tended to have a functional transcriptome similar to alpha-cells. However, a shortcoming of transcriptomic analysis alone is that some dispersed human alpha-cells contained a high insulin mRNA content that was not translated into insulin [8], reinforcing the importance of considering both transcriptome and proteome when assigning cell phenotype. A deep proteomic analysis of single cells within sections of adult human pancreas identified twelve endocrine sub-types, including those with cell surface markers for both alpha/beta-cells, delta/beta-cells, and alpha/delta-cells [9]. The highest frequency of alpha/beta-cells was found in a sub-population of small sized islets. Functional proteomic analysis revealed that both beta-cells and some beta/delta-cells were engaged in TCA cycle, fatty acid and glucose metabolism, suggesting that the bi-hormonal cells exhibited beta-cell-like function. Alpha/beta-hybrid cells expressed progenitor cell and cell cycle proliferation markers suggesting the potential for developmental plasticity. These data suggest that in the normal adult pancreas there are bi-hormonal cell sub-populations that are both metabolically functional and have trans-lineage plastic potential.

Bi-hormonal Cells as a Residue of Fetal Development

One explanation of the presence of bi-hormonal cells postnatally is that they represent a vestige of the developmental pathways from which islet endocrine cell lineages derived *in utero*. Pancreas development commences with the expression of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) in the foregut dorsal endoderm in two ventrolateral regions, which then undergo morphogenesis to create pancreatic rudiments [10]. An orchestrated cascade of transcription factor expression follows to convert primitive endoderm into the dorsal and ventral pancreatic buds [11]. Around embryonic day 9.5 to 10.5 in the mouse fusion of the dorsal and ventral pancreas rudiments occurs, and a branching morphogenesis follows to create a ductal tree from which both exocrine and endocrine cell lineages are generated. Beta-and other endocrine cell progenitors develop from the mid-region epithelium of each primitive duct and, accompanied by endothelial cells that produce the islet basement membrane, migrate into the parenchyma and self-assemble into individual islets, each with a specialist vascular bed (**Figure 1**). Glucagon/insulin bi-hormonal cells are seen within the human fetal pancreas at weeks 6-7 of gestation but disappear during later islet maturation [12]. This ontogeny is supported by studies *in vitro* of the differentiation of alpha and beta-cells from human embryonic stem cells where insulin/glucagon bi-hormonal cells appear coincident with the appearance of the endocrine cell populations [13]. The functional maturation of beta-cells *in vivo* occurs prior to birth in human but between birth and weaning in the mouse [14].

Single cell RNAseq analysis of the human fetal pancreas showed that bi-hormonal cells have a transcriptional profile more similar to alpha-cells than beta-cells [7]. Beta-like cells generated *in vitro* from human embryonic stem cells contain a bi-hormonal cell population twice that of insulin mono-hormonal cells [15]. However, most of these cells were clustered around alpha-cells and expressed very low levels of insulin. Despite the apparent absence of a distinct functional transcriptional profile in fetal bi-hormonal cells there is substantial plastic potential, and fetal islet cells committed to the synthesis of one hormone can give rise of different mono-hormonal cells in the adult pancreas [16]. The phenotype of bi-hormonal cells may represent a weakened timing or magnitude of expression of transcription factors that are signatures for each defined endocrine cell type. During islet differentiation within the fetus most transcription factors both induce a dominant endocrine cell phenotype while also suppressing transcription factors defining other endocrine cell types. An example of this is a sub-optimal expression of Foxo1in beta-cells which results in cells that are multi-hormonal [17]. Thus, a

residual population of bi-hormonal cells in the islets following birth may simply represent the transcriptional outliers of the endocrine lineage separation process *in utero* that did not become definitive mono-hormonal cells.

Bi-hormonal Cells as a Regenerative Mechanism

Why then might a bi-hormonal cell population be retained into adult life? One possibility is that they represent a regenerative reserve of endocrine cells with plastic lineage capacity to address a sub-optimal endocrine cell balance during times of metabolic stress. The lineage commitment of both alpha- and beta-cells is relatively epigenetically shallow and can be altered by the experimental ectopic expression of a relatively small number of transcription factors. Human donor islets transfected with the transcription factors PDX1 and MAF BZIP Transcription Factor A (MAFA), which contribute to the beta-cell phenotype, underwent alpha- to beta-cell transdifferentiation following transplant into diabetic mice, as did alpha-cells depleted of ARX expression, a key gene necessary for alpha-cell function [18]. Over-expression of the beta-cell transcription factor Paired Box 4 (Pax4) caused the conversion of alpha-cells into insulin-producing cells in a mouse model [19], with similar findings following transfection of human islets from subjects with type 2 diabetes [20]. Hence, naturally occurring bi-hormonal cells may be primed for a rapid phenotypic conversion as a result of prolonged hyperglycemic stress.

We observed that bi-hormonal cells are often located towards the rim of both mouse and human islets [2] which has been proposed as a niche for resident beta-cell progenitors [21,22]. It is possible, therefore, that this also represents a preferred location for endocrine cell transdifferentiation. Since the islet rim is an area of extensive juxtaposition of alpha-, beta-, delta- and epsilon-cells it represents a site of high paracrine cell interactions, for instance the availability of alpha-cell-derived GLP-1.

A pool of resident, phenotypically plastic bi-hormonal cells would be advantageous as the regenerative capacity of beta-cells by cell replication alone is extremely low both in humans and in animal models beyond early childhood [23,24]. There remains a sub-population of progenitor beta-cells within islets following birth, which express insulin but exhibit poor GSIS, due to a paucity of glucose receptor-2 (GLUT2) expression [25,26], their abundance declines rapidly after puberty [27]. The possibility that these are derived from bi-hormonal cells seems unlikely since they are transcriptionally distinct by scRNA-seq, at least in the mouse pancreas [28]. Similarly, other sub-populations of insulin-expressing cells such as the hub beta-cells that coordinate pulsing of intracellular calcium levels and first-phase insulin release are transcriptionally different from bi-hormonal cells [29].

In the face of the poor ability of beta-cells to proliferate in

adult life a functional increase in insulin availability in response to insulin resistance might be achieved by lineage plasticity of islet endocrine cells, and an increase in bi-hormonal cell abundance (**Figure 1**). In support of this concept the number of insulin-immunoreactive cells also containing either glucagon or somatostatin in human islets was 2-3-fold greater in donors with type 2 diabetes than in metabolically healthy controls [30–32]. This may boost insulin secretion capacity and counteract insulin resistance in type 2 diabetes [17]. Arguing against such a direct relationship with insulin demand was a lack of correlation seen between bi-hormonal cell number and basal or first-phase insulin secretion, or the total insulin secretion during a hyperglycemic clamp [33]. This would suggest that bi-hormonal glucagon/insulin cells are not themselves likely to greatly contribute to GSIS but are more likely to represent an intermediate step in a more complete transdifferentiation into functional beta-cells under metabolic stress.

To what extent is transdifferentiation to beta-cells possible in response to hyperglycemic stress alone? Following a rapid induction of hyperglycemia in mice using the beta-cell toxin, streptozotocin, cell lineage tracing demonstrated a prompt increase in glucagon/insulin bi-hormonal cells, but only around 10% of these became mono-hormonal insulin-expressing cells over a period of three weeks [34]. This equates to approximately 2% of islet alpha-cells that were capable of full transdifferentiation, but this was sufficient to enable a partial recovery of beta-cell mass. Pharmacologic inhibition of the glucagon receptor activation was able to reverse diabetes in type 1 diabetic mice with an estimated 15% of new beta-cells being derived from alpha-cells through transdifferentiation assessed by lineage tracking [35]. Transcriptional and epigenetic changes in islet endocrine cells have been followed longitudinally during the induction of diabetes in mice exposed to a high fat diet [36]. Intercellular communication between islet cells, and between islet cells and the vascular bed or with macrophages were all modified, and the population of alpha- and delta-cells both relatively declined compared to beta-cells. The presence of several bi-hormonal cell populations changed in abundance during the development of hyperglycemia, including insulin/somatostatin and glucagon/somatostatin cells. Two populations of alpha-cells were identified: one retaining strong alpha-cell-related functional activity and the other showing reduced glucagon expression and a more beta-cell transitional phenotype. Three populations of delta-cells were found in the progression towards diabetes: the first retaining a strong delta-cell transcriptional profile, a second showing a transitional profile towards beta-cells with both INS1 and INS2 expression, and the third beginning to express alpha-cell transcriptional markers. These findings highlight multiple islet cell transcriptional responses when faced with hyperglycemic and hyperlipidemic stress with sub-populations of alpha- and delta-cells increasingly adopting beta-cell characteristics.

However, adult beta-cells can be experimentally manipulated to re-enter the proliferative cycle outside of metabolic stress. Adult human beta-cells reinitiated proliferation both *in vitro* and when grafted into mice *in vivo* following exposure to the Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) inhibitor, harmine, together with a GLP-1 receptor agonist [37]. Subsequent analysis showed that DYRK1A inhibitors activated a sub-population of alpha-cells to proliferate, adopt a glucagon/insulin bi-hormonal phenotype, and undergo transdifferentiation to become beta-cells capable of GSIS [38]. A variety of other pharmacological interventions have also been found to induce beta-cell mass through transdifferentiation (**Table 1**). Alpha- to beta-cell transdifferentiation was enhanced in human islets by exposure to GLP-1 alone [4] and in rodent islets following administration of gamma aminobutyric acid (GABA) [39], which resulted in a downregulation of the transcription factor Aristaless Related Homeobox (Arx) in alpha-cells. Serotonin treatment increased the percentage of glucagon/insulin bi-hormonal cells along with beta-cell-specific transcription factors such as NK6 Homeobox 1 (Nkx6.1) in both isolated mouse and human islets, and *in vivo* in mice fed a high fat diet as a model for type 2 diabetes [40]. GLP-1, GABA and serotonin are each paracrine trophic beta-cell factors released from alpha-cells (GLP-1) or beta-cells (GABA and serotonin) during hyperglycemic stress [41]. When considering a possible therapeutic strategy, the SGLT2 inhibitor, dapagliflozin, used in the control of T2D also promoted alpha- to beta-cell transdifferentiation in diabetic mice [42].

Islet cell trandifferentiation to enhance the beta-cell population is not limited to alpha-cells since a bi-hormonal phenotype expressing both somatostatin and insulin was identified during induced transdifferentiation of delta- to beta-cells following the destruction of beta-cells in the zebrafish [43] (**Figure 1**). Similarly, an experimental loss of beta-cells in pre-weaning mice caused a subsequent regeneration by transdifferentiation from delta-cells, but this pathway was reduced substantially as the animals became adult when alpha- to beta-cell transdifferentiation become more frequent [44]. The somatostatin/insulin bi-hormonal cell population observed in early life may be transient and derived from pancreatic duct epithelium since Neurogenin-3 (Ngn3)-expressing ductal endocrine progenitors' express somatostatin and subsequently transdifferentiate into beta-cells [45].

A negative correlation exists between increasing age and islet cellular plasticity [33,46] which, if bi-hormonal cells represent an intermediary mechanism towards beta-cell expansion might reflect their decreased abundance. The relative abundance of alpha-cells in human islets increases with age until around 30 years, whilst over the lifespan delta-cell presence decreases with age [31]. We found that the proportion of beta-cells per islet increased during childhood to reach adult values around the time of puberty, but thereafter alpha to beta-cell ratio remained constant [2]. Histological analysis has shown that beta-cell mass increases thirty-fold between birth and adulthood [47], with the growth velocity being greatest between birth and 2 years age [33], after which the beta-cells become proliferatively quiescent [48]. With respect to bi-hormonal cell ontogeny, as stated previously, we found a negative relationship between insulin/somatostatin and glucagon/somatostatin bi-hormonal cells in the human pancreas, but glucagon/insulin cell abundance increased with age [2]. This suggests that the decrease in islet cell regenerative capacity with age is not directly attributable to a loss bi-hormonal cell.

Bi-hormonal Cells Reflect Dedifferentiation for Beta-cell Survival

An increase in bi-hormonal cell abundance during hyperglycemia could represent an attempt to increase insulin secretion capacity, but it might also represent a dedifferentiation of beta-cells to an alpha-cell phenotype as a result of metabolic stress, as seen in type 2 diabetes [17] (**Figure 1**). Beta-cell dedifferentiation has also been observed as part of normal aging [46]. Beta-cell mass is lost during type 2 diabetes, in part through apoptosis in response to gluco- and lipotoxicity. However, the rate of apoptosis observed in animal models is insufficient to fully account for the beta-cell loss. It is thought that the predominant mechanism to account for loss of beta-cells is dedifferentiation either to a hormone-null, degranulated progenitor endocrine-like state [17], or a transdifferentiation to adopt an alpha-cell phenotype [49]. A similar dedifferentiation of beta-cells has been reported to occur in type 1 diabetes also and may represent a survival mechanism to avoid autoimmune destruction, especially if the phenotypic change is potentially reversible [50].

Beta-cell dedifferentiation is associated with a relative loss of expression of genes defining beta-cells, such as the transcription factors Pdx1, Nkx6.1 and MafA [51,52]; the appearance of lineage precursor-associated gene expression such as Ngn3, Nanog and aldehyde dehydrogenase 1 family member A3 (Aldh1a3) [53], and the expression of genes associated with alpha-cells that would normally be repressed in beta-cells such as proglucagon and Arx [54] (**Table 1**). Some beta-cells may progress to a hormone-negative phenotype, others transdifferentiating into an alpha-cell phenotype, and some simply becoming bi-hormonal, depending on the extent and duration of metabolic stress. Presently, there is a lack of a distinct transcriptional signature for bi-hormonal cells that can distinguish between alpha-cells transdifferentiating to become beta-cell versus beta-cells dedifferentiating and adopting an alpha-cell phenotype.

Importantly for therapeutic strategies the beta-cell dedifferentiated state appears to be reversible. Early
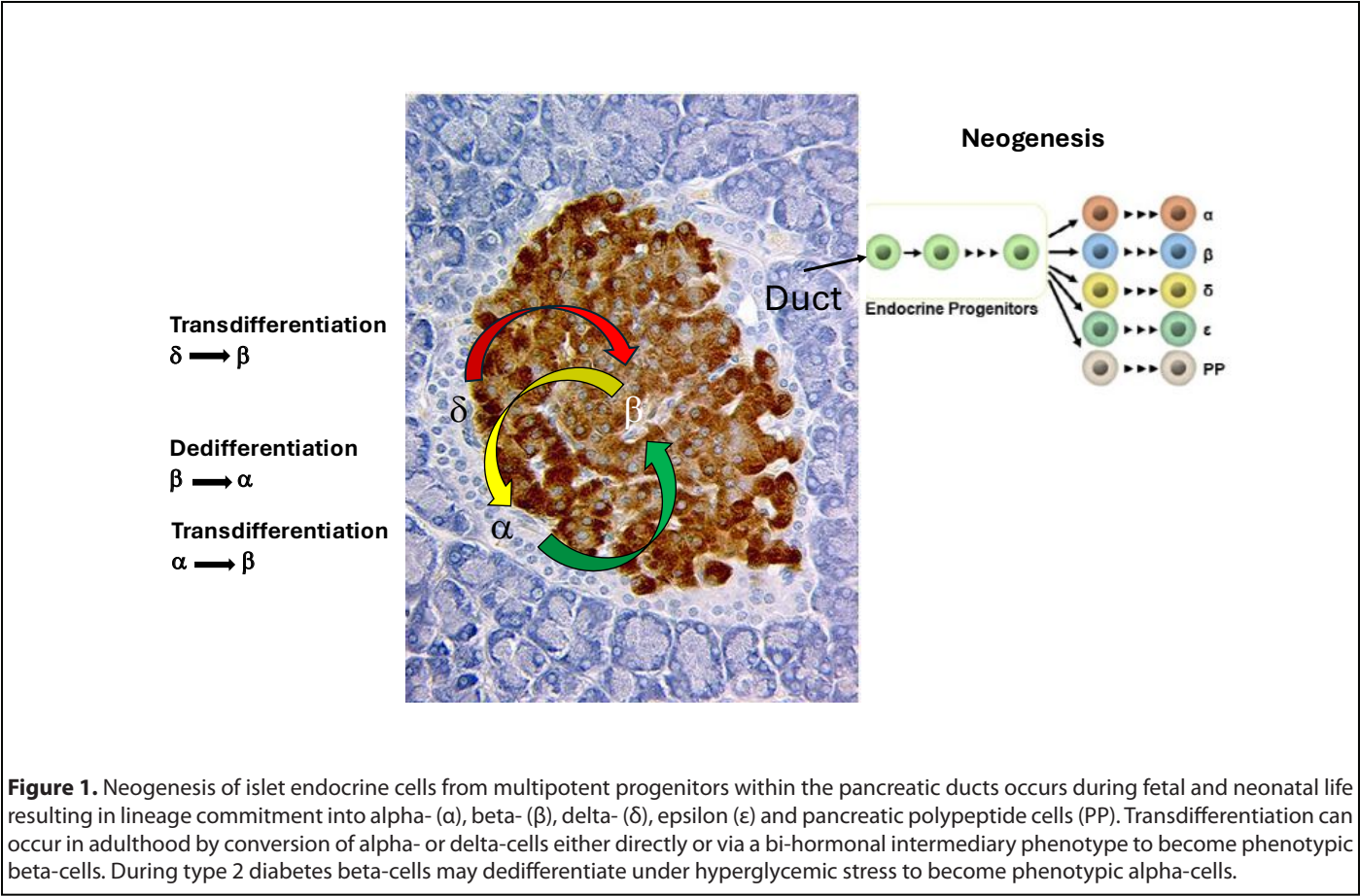
Figure 1. Neogenesis of islet endocrine cells from multipotent progenitors within the pancreatic ducts occurs during fetal and neonatal life resulting in lineage commitment into alpha- (α), beta- (β), delta- (δ), epsilon (ε) and pancreatic polypeptide cells (PP). Transdifferentiation can occur in adulthood by conversion of alpha- or delta-cells either directly or via a bi-hormonal intermediary phenotype to become phenotypic beta-cells. During type 2 diabetes beta-cells may dedifferentiate under hyperglycemic stress to become phenotypic alpha-cells.

Table 1. Comparison of experimental models, gene and protein markers, triggers of lineage transition, and the functional capacity of the resulting cells reported in the literature for transdifferentiation of alpha- to beta-cells, or dedifferentiation of beta- to alpha-cells. STZ: Streptozotocin, targeted beta-cell expression of diphtheria toxin; PC1/3: Prohormone Convertase 1/3; MafA: MAF bZIP transcription factor A; GLUT2: Glucose Transporter 2; GLP-1: Glucagon-Like Peptide-1; GABA: Gamma-Aminobutyric Acid; SGLT2: Sodium/Glucose Cotransporter 2; DYRK1A: Dual Specificity Tyrosine-Phosphorylation-Regulated Kinase 1A; Arx: Aristaless related homeobox; Aldh1a3: Aldehyde dehydrogenase 1 family member A3.

	Transdifferentiation α → β	Dedifferentiation β → α
Experimental models	β-cell loss (STZ, Diphtheria toxin)	High fat diet, hyperglycaemia
Gene and protein markers	Proinsulin, PC1/3, MafA, GLUT2	Proglucagon, Arx, Aldh1a3
Lineage transition triggers	GLP-1, GABA, Serotonin SGLT2 inhibition DYRK1A inhibition	Type 2 diabetes
Functional capacity	Immature first phase insulin release, reduced insulin content	Glucose-sensitive glucagon release

intervention in type 2 diabetes with insulin together with metformin improved beta-cell function with long-term diabetes remission [55]. In the *db/db* mouse model of diabetes calorie restriction was able to reverse beta-cell dedifferentiation [56], while cellular therapy though the grafting of umbilical cord-derived mesenchymal stem cells was also able to reverse engineer dedifferentiation through the re-expression of transcription factors such as Pdx1 [57].

Conclusion

Islet bi-hormonal cells are present as a sub-population of endocrine cells throughout life, first appearing as part of the lineage development of the endocrine pancreas during fetal organogenesis. There is extensive phenotypic diversity throughout the islet endocrine cell population which persists into adulthood. While there are limited evidence that bi-

hormonal cells themselves add an additional element of glycemic control compared to mono-hormonal islet cells, they can undergo transdifferentiation to become functional beta-cells under conditions of hyperglycemic stress. This involves a transcriptional realignment whereby beta-cell-specific genes are upregulated and alpha- or delta-cell associated genes are repressed and are under epigenetic control. Some bi-hormonal cell populations are also proliferative, thereby additionally expanding the potential for transdifferentiation. Transdifferentiation can be enhanced in response to therapeutic agents such as GLP-1 receptor agonists or serotonin. However, bi-hormonal cells might also result from a dedifferentiation of beta-cells during diabetes to avoid cellular stress and death. The balance between a capacity for transdifferentiation to yield new beta-cells and a loss of beta-cell function through dedifferentiation is not understood but is likely to be influenced by the degree and duration of metabolic stress. Can the transdifferentiation of bi-hormonal cells be considered as a viable therapeutic option to prevent or control diabetes? It may already be contributing to the actions of commonly used drugs for glycemic control such as GLP-1 receptor agonists or metformin.

Key information is still lacking to enable transdifferentiation of non-beta islet cells into functional beta-cells *in vivo* as a potential therapeutic strategy to delay or prevent the onset of diabetes. Firstly, it is unclear the extent to which transdifferentiation gives rise to fully functional, glucose-sensitive beta-cells. Whilst some information can be gleaned from transcriptomic and proteomic analysis of single transdifferentiated cells in situ, specific molecular biomarkers will be needed to bulk separate transdifferentiated from 'wild type' beta-cells for detailed kinetic analysis of glucose-stimulated insulin secretion. Secondly, whilst a number of small molecules have been suggested to initiate alpha- to beta-cell transdifferentiation many of these are repurposed drugs with widespread additional actions throughout the body. Effective dosage in enabling islet cell transdifferentiation versus other actions will require detailed pharmacokinetic analysis in a standardized animal model. However, the accumulating findings that islet cells can adopt a spectrum of overlapping lineage phenotypes provides impetus for further in-depth study.

Acknowledgements

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