



Systematic single-cell analysis provides new insights into heterogeneity and plasticity of the pancreas

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ABSTRACT

Background: Diabetes mellitus is characterized by loss or dysfunction of insulin-producing β -cells in the pancreas, resulting in failure of blood glucose regulation and devastating secondary complications. Thus, β -cells are currently the prime target for cell-replacement and regenerative therapy. Triggering endogenous repair is a promising strategy to restore β -cell mass and normoglycemia in diabetic patients. Potential strategies include targeting specific β -cell subpopulations to increase proliferation or maturation. Alternatively, transdifferentiation of pancreatic islet cells (e.g. α - or δ -cells), extra-islet cells (acinar and ductal cells), hepatocytes, or intestinal cells into insulin-producing cells might improve glycemic control. To this end, it is crucial to systematically characterize and unravel the transcriptional program of all pancreatic cell types at the molecular level in homeostasis and disease. Furthermore, it is necessary to better determine the underlying mechanisms of β -cell maturation, maintenance, and dysfunction in diabetes, to identify and molecularly profile endocrine subpopulations with regenerative potential, and to translate the findings from mice to man. Recent approaches in single-cell biology started to illuminate heterogeneity and plasticity in the pancreas that might be targeted for β -cell regeneration in diabetic patients.

Scope of review: This review discusses recent literature on single-cell analysis including single-cell RNA sequencing, single-cell mass cytometry, and flow cytometry of pancreatic cell types in the context of mechanisms of endogenous β -cell regeneration. We discuss new findings on the regulation of postnatal β -cell proliferation and maturation. We highlight how single-cell analysis recapitulates described principles of functional β -cell heterogeneity in animal models and adds new knowledge on the extent of β -cell heterogeneity in humans as well as its role in homeostasis and disease. Furthermore, we summarize the findings on cell subpopulations with regenerative potential that might enable the formation of new β -cells in diseased state. Finally, we review new data on the transcriptional program and function of rare pancreatic cell types and their implication in diabetes.

Major conclusion: Novel, single-cell technologies offer high molecular resolution of cellular heterogeneity within the pancreas and provide information on processes and factors that govern β -cell homeostasis, proliferation, and maturation. Eventually, these technologies might lead to the characterization of cells with regenerative potential and unravel disease-associated changes in gene expression to identify cellular and molecular targets for therapy.

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Keywords β -Cell heterogeneity; Single-cell analysis; Diabetes; Regeneration; Endocrine cells; Transdifferentiation; Dedifferentiation; Maturation; Subpopulations

1. INTRODUCTION

Diabetes mellitus is a complex and multifactorial disease characterized by progressive loss or dysfunction of the insulin-producing β -cells in the pancreas. This results in chronic hyperglycemia and systemic metabolic complications and, in the long-term, causes multi-organ damage including nephropathy, retinopathy, and enteropathy. Today, over 382 million people worldwide have been diagnosed with diabetes and the number is expected to rise to 592 million by 2035 [1]. Type 1 diabetes (T1D) is an autoimmune disorder caused by destruction of β -

cells through cytotoxic T-cells. Unlike in T1D, onset of the more prevalent type 2 diabetes (T2D) is usually in adulthood and is often consequence of genetic predisposition, obesity and lack of physical exercise. T2D is triggered by insulin resistance of the peripheral tissues, which is concomitant with β -cell mass expansion, β -cell exhaustion, and gradual loss of functional β -cell mass through β -cell dedifferentiation and/or β -cell death [2,3]. Thus, the common feature of both pathologies is loss of functional β -cells. Despite its high prevalence and increasing impact on global health, diabetes is still incurable and our knowledge of the underlying pathomechanisms is far

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from complete. Current treatments succeed in reducing symptoms; however, they fail to alleviate long-term complications and require lifelong compliance from patients. Therefore, intensive efforts in the field of diabetes research are put into the development of novel therapeutic strategies to stop the progression of the disease and restore functional β -cell mass.

Human islet transplantation from cadaveric donors has been successfully established as a therapeutic treatment for a subset of patients with “brittle” T1D that do not respond to standard conventional and intensive insulin therapies and suffer from kidney failure [4,5]. However, donor shortage and risks associated with life-long immunosuppression demand the development of alternative therapies. Two main strategies are currently extensively explored to replace lost and/or dysfunctional β -cells: i) *in vitro* differentiation of β -cells from stem cells and ii) endogenous β -cell regeneration. The former holds great promise for cell-replacement therapy and tissue engineering. In the past years, major advances have enabled the generation of monohormonal and glucose-responsive β -like cells from human embryonic stem cells and patient-derived induced pluripotent stem cells [6–8]. Importantly, these cells were able to secrete insulin and restored normoglycemia in diabetic mice [9]. Still, prior to application in humans, the differentiation efficiency and functionality of *in vitro* generated β -like cells needs to be improved. In this regard, the field would benefit greatly from a better understanding of the postnatal β -cell maturation process and the identification of biomarkers that label the different maturation stages and functional glucose-responsive β -cells. In addition, their immune-protection as well as safety must be guaranteed as not fully differentiated stem cells might have teratoma-initiating potential.

Stimulating regeneration of insulin-producing cells from cells residing within the adult pancreas or even in other metabolically active organs,

such as the liver or gut (not discussed in this review), is an appealing approach that could bypass the aforementioned hurdles. The main routes pursued to restore functional β -cell mass *in situ* include boosting the replication of remaining β -cells, maturation of immature (dedifferentiated) β -cell subpopulations, mobilization of putative precursors present in the adult pancreas and reprogramming of other cell types into insulin-producing β -like cells (Figure 1) [10]. Important in this respect is the existence of β -cell subpopulations that differ in their glucose responsiveness, proliferative activity, maturation state, or susceptibility to metabolic deregulation in animal models [11]. Moreover, adult exocrine and other endocrine cell types showed the ability to reprogram and produce insulin under certain conditions [12]. Further characterization of these candidate sources for the generation of new insulin-producing cells as well as the identification of biomarkers and therapeutic targets requires detailed dissection of the cellular heterogeneity within the pancreas and their underlying molecular mechanisms. To this end, single-cell studies might be paradigm changing. Single-cell technologies allow for simultaneously measuring the expression of tens to thousands of genes (e.g. single-cell RNA sequencing) or proteins (e.g. single-cell mass cytometry, flow cytometry) in individual cells with high-throughput and precision. Clustering of cells as per their expression profiles allows for unbiased detection and characterization of cell types and states including rare or unanticipated subpopulations that are masked in bulk analyses (Figure 2). By pooling many cells with partially correlated measurements, one can derive rich molecular profiles without prior knowledge of defining criteria and screen for subtype specific marker genes even if only a limited number of transcripts or proteins per cell are captured [13,14]. In addition, single-cell measurements provide an accurate temporal resolution of continuous processes, such as differentiation or reprogramming, as cells of all present (transient and stable) stages are

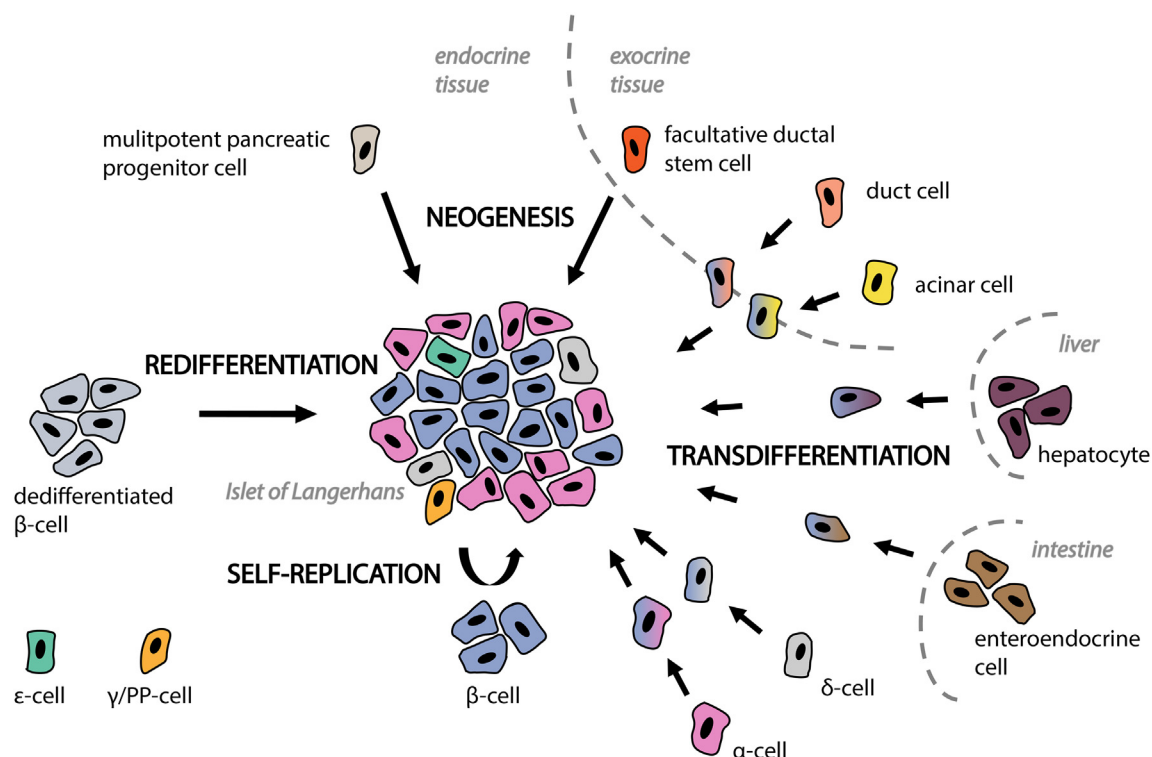
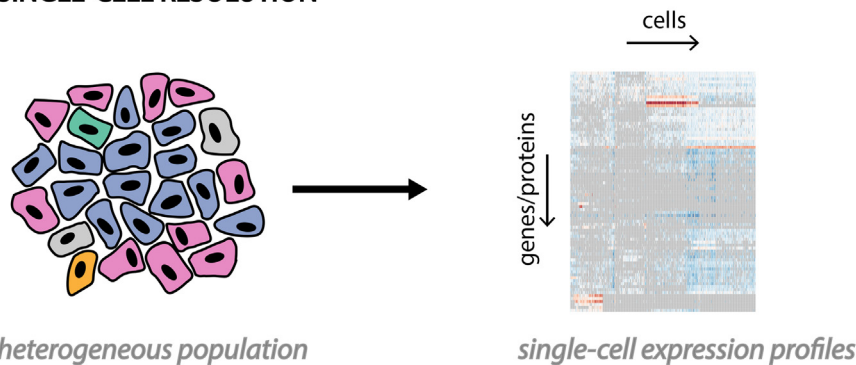
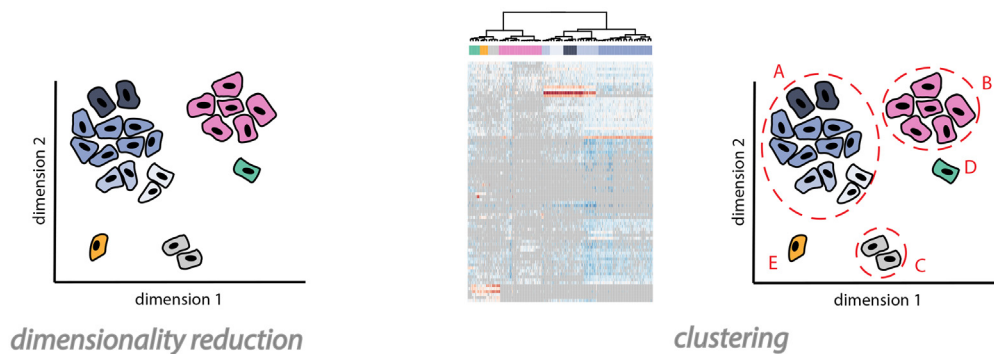


Figure 1: Main routes to restore functional β -cell mass *in situ*. Schematic summarizing the possible ways of β -cell regeneration that are discussed in the text.

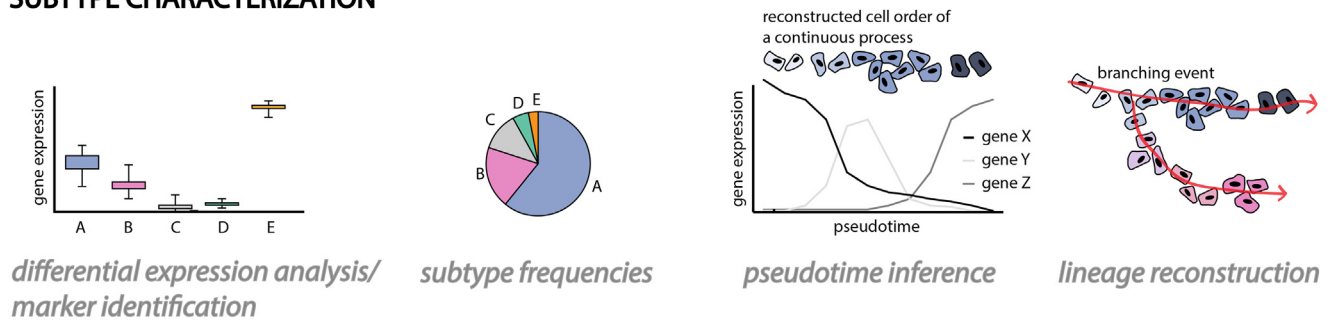
SINGLE-CELL RESOLUTION



VISUALIZATION AND SUBTYPE IDENTIFICATION



SUBTYPE CHARACTERIZATION



SUBTYPE-SPECIFIC ALTERATIONS IN DISEASE

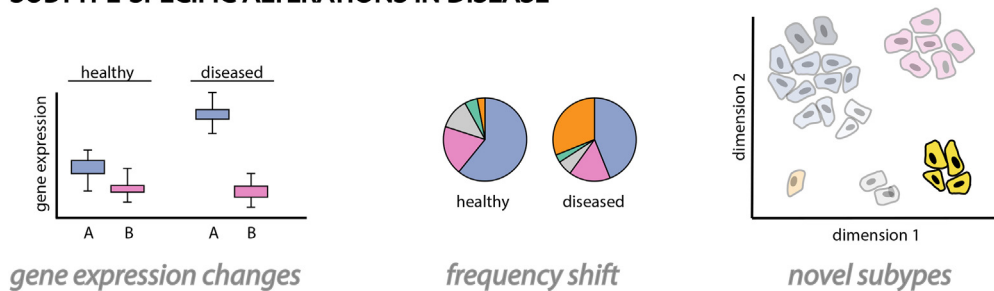


Figure 2: Identification and characterization of heterogeneity by single-cell analysis. Single-cell approaches enable the identification and characterization of cellular subtypes in a heterogeneous population and their implications in disease based on rich expression profiles acquired at single-cell resolution. Computational analyses translate the high-dimensional data into low-dimensional cell maps and extract information on subtype composition and gene expression differences within and between subtypes in healthy and diseased conditions as well as reconstruct cellular trajectories of continuous processes.

captured simultaneously. The temporal order and lineage choices can be reconstructed from single-cell snapshot data using computational algorithms that infer a pseudotime and detect branching events [15–17]. This provides information on the genes most involved in determining the identity of a cell and on the factors that are expressed transiently. Finally, single-cell analyses have important implications in medicine since they allow parallel identification of disease-associated alterations in cellular composition as well as in gene expression of cell subtypes. Differences shed light on how cell-to-cell variability could lead to different cell function or fate of seemingly identical cells of a (sub)population, and on how these subpopulations respond to external cues or drugs, and therefore on etiology, pathomechanisms, and treatment efficiency (Figure 2).

Here we provide an overview of single-cell analyses of pancreatic cells and discuss how these new data add to our knowledge of pancreatic cell heterogeneity and improve our understanding of possible endogenous β -cell regeneration routes.

2. DETERMINATION OF THE CELLULAR COMPOSITION OF THE PANCREAS

The mature pancreas can be subdivided into the functionally distinct endocrine and exocrine compartment. The exocrine tissue comprises more than 95% of the pancreas mass and is composed of acinar and ductal cells. The endocrine tissue is organized into cell clusters, the so-called islets of Langerhans, that are dispersed within the exocrine tissue. The islets of Langerhans are comprised of five distinct endocrine cell types: α -cells (secreting glucagon), β -cells (insulin), γ /PP-cells (pancreatic polypeptide), δ -cells (somatostatin), and ϵ -cells (ghrelin), together making up less than 5% of the pancreas mass.

Islet composition is determined during embryonic development, and has been extensively discussed in excellent reviews [18,19]. Briefly, in mice, the pancreas is first identified by the expression of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) in the foregut endoderm at embryonic day (E) 8.5. Pdx1 expression is followed by the expression of several other transcription factors that are required for pancreatic specification, including Pancreas specific transcription factor 1 (Ptf1a), Forkhead box protein A 1/2 (Foxa1/2), SRY box 9 (Sox9), Hepatocyte nuclear factor-1 β (Hnf1 β), Gata4/6, Hairy and enhancer of split 1 (Hes1), and Nkx2.2 [18,19]. During the initial phase of murine pancreatic development, which has been referred to as primary transition, the Pdx1 multipotent pancreatic progenitor cells (MPCs) have high proliferative capacity and primarily give rise to a few glucagon-producing α -cells [20,21]. The secondary transition, which encompasses the time between E12.5 and birth, is characterized by expansion and branching morphogenesis of the pancreatic epithelium and is closely linked to endocrine cell differentiation. During these complex morphogenetic events, MPCs become lineage restricted and adopt either tip or trunk identity. The trunk progenitors are bi-potential and further differentiate into ductal or endocrine precursor cells. The ductal versus endocrine fate decision is thought to be controlled by graded Notch activity, with high Notch signaling promoting ductal fate and localized inhibition of Notch signaling, allowing transient Ngn3 expression and endocrine differentiation [22]. Shortly after induction of Ngn3 expression, endocrine committed cells start to delaminate from the ductal epithelium and form nascent islets. Ngn3 activates the expression of several transcription factors implicated in endocrine lineage maintenance and specification including the pan-endocrine marker neuronal differentiation 1 (Neurod1), the LIM homeobox protein islet 1 (Isl1), paired box 4 and 6 (Pax4 and Pax6), and aristaless-related homeobox (Arx).

Specification into α - and β -cell precursor relies on mutual repression between opposing lineage determinants. The transcription factors Pax4, Nkx2.2, and Nkx6.1 are direct transcriptional repressors of Arx, which specifies α -cell fate and conversely, Arx represses Pax4, Nkx6.1, and Pdx1, which specify β -cell fate [23]. The subsequent maturation of insulin-positive cells into functional, glucose-responsive β -cells is characterized by a switch from MafB to MafA expression in mice [24–26]. Importantly, the transcription factors Pax6 and Nkx2.2 are not only crucial for β -cell lineage formation and function but they also actively repress non- β -cell transcriptional programs, a requirement for maintenance of β -cell identity [27,28]. While the transcription factors determining α - and β -cell fate are known, the formation of the less abundant γ /PP-, δ - and ϵ -cells is not well understood and their fate might be determined by combinatorial action of different transcription factors.

Physiologically, α - and β -cells act in concert to regulate blood glucose levels. β -cells release insulin in response to high blood glucose levels to stimulate glucose uptake into peripheral tissues such as liver, muscle, and fat tissue. In contrast, α -cells release glucagon in response to low levels of glucose to stimulate glycogenolysis in the liver and in order to prevent hypoglycemia during fasting or exercise [29]. So far, the function of δ -, γ /PP-, and ϵ -cells remains largely elusive due to their low abundance. Recently, it has been shown that the peptide hormone urocortin3 which is co-released with insulin potentiates somatostatin secretion from δ -cells and possibly in this way δ -cells fine-tune insulin secretion [30]. Pancreatic polypeptide, which is released by γ /PP-cells, is believed to regulate pancreatic exocrine and endocrine secretions [31–33].

Interestingly, differences in islet composition and architecture between rodents and humans have been reported. Murine islets of Langerhans are comprised of up to 60–80% of insulin-producing β -cells that are clustered in the center of the islet and are surrounded by a mantle of endocrine α - (15–20%), δ - (< 10% of islet cells), and γ /PP-cells (< 1% of islet cells) [34]. Cell type composition and architecture of human islets seem to be more variable and are still matter of debate [35–37]. Yet, after birth, the majority is similarly organized as murine islets and show the characteristic mantle-core feature [37]. The human islet cell-type composition is on average ~50% β -cells, ~40% α -cells, and ~10% δ -cells, up to a few percent γ /PP cells, and very few ϵ -cells [38–40]. The differences between rodents and humans in islet cell-type abundance might indicate additional functions of α -cells and/or increased functional dependencies of α - and β -cells in humans.

3. SINGLE-CELL ANALYSIS UNRAVELS THE TRANSCRIPTIONAL PROGRAM OF ADULT PANCREATIC CELL TYPES AND IDENTIFIES SPECIES-SPECIFIC DIFFERENCES

Recent technological advances in the field of single-cell ‘omics’ now allow for easily measuring gene or protein expression in thousands of cells. Importantly, pure cell populations can be derived during subsequent data analyses by clustering of cells based on their high-dimensional expression profiles, which is not possible through isolation from a population using a few markers. The systematic transcriptome analysis of adult human pancreatic cell types revealed that the expression of most lineage-determining transcription factors is conserved between human and mice (Figure 3). Strikingly, classifying hormone expression accounted for 50% of the total cellular transcripts in β -, γ /PP-, and δ -cells. Whereas in α - and ϵ -cells the expression of *GCG* and *GHRL*, respectively, made up 20% of their transcriptomes [41]. Single-cell analysis also revealed that 15% of all α - and β -cell enriched genes show distinct species-dependent expression [42].

pan-endocrine: *PAX6, MORF4L1, NEUROD1, INSM1, NKX2.2*

β-cell	α-cell	ε-cell	δ-cell	γ/PP-cell
<i>PAX4</i>	<i>ARX</i>	<i>GHRLOS</i>	<i>HHEX</i>	<i>ARX</i>
<i>PDX1</i>	<i>MAFB</i>	<i>NPY1R</i>	<i>ETV1</i>	<i>ETV1</i>
<i>MAFA</i>	<i>IRX2</i>	<i>ASGR1</i>	<i>MAFB</i>	<i>UCN3</i>
<i>MAFB</i>	<i>GC</i>	<i>ARX</i>	<i>LEPR</i>	<i>FEV</i>
<i>BMI1</i>	<i>FEV</i>		<i>GHSR</i>	
<i>DLK1</i>	<i>TM4SF4</i>		<i>GPR120</i>	
<i>SIX2/3</i>	<i>GPR119</i>		<i>POUF3F1</i>	
<i>ID1</i>	<i>UCN3</i>			
<i>IAPP</i>	<i>FEV</i>			
<i>UCN3</i>	<i>DPP4</i>			
<i>OLIG1</i>				

Figure 3: Transcriptional program of human pancreatic endocrine cells revealed by single-cell transcriptomics. Depicted genes are highly or exclusively expressed in the given endocrine cell type. Genes highlighted in blue show species-specific differences in cell-type expression.

Genes with robust expression in human, but not in mouse β-cells are for example *RBP4*, *DLK1*, *ADCYAP1*, *RGS16*, *SOX4*, *BMP5*, *TIMP2*, *TSPAN1*, *MAFB*, and *TFF3*. Genes with high expression in mouse but not in human β-cells are *UCN3*, *S100A1*, *ADH1C*, *FAM151A*, *COX6A2*, *BACE2*, *TTYH2*, *SYTL4*, *SDF2L1*, *FRZB*, and *PRLR* [42].

Besides differences in expression levels, some cell-type enriched genes also showed notable species differences. As mentioned above the transcription factors MafB and MafA regulate β-cell lineage formation and function in rodents [26]. During mouse endocrine lineage specification MafB expression becomes restricted to α-cells, whereas MafA is expressed in β-cells [24–26]. In contrast, in humans, *MAFB* expression has been detected in adult α-cells but also in β-cells and its expression pattern has now been expanded to δ-cells [41–44]. Interestingly, Li et al. found that half of the β-cells they studied expressed *MAFA* and *MAFB* concomitantly, suggesting that *MAFB* is a new marker describing β-cell heterogeneity in humans [44]. The Notch pathway component *delta like non-canonical Notch ligand 1* (*DLK1*), which has been associated with T1D in genome-wide association studies (GWAS), was found to be specifically expressed in human β-cells, whereas it is highly expressed in postnatal, immature α- and β-cells, and adult α-cells but not adult β-cells in mice [42,44–46]. Similarly, several groups confirmed previous findings by the Kaestner group that the gene *group-specific component (vitamin D binding protein)* (*GC*) is specifically expressed in α-cells in human tissue, whereas it is co-expressed with insulin in mouse islets (Figure 3) [44,47,48]. Vitamin D deficiency and common *GC* non-coding variants have been associated with T1D, T2D, and an increased risk of gestational diabetes mellitus [49,50]. These findings implicate a contribution of dysfunctional α-cells to the pathogenesis of diabetes. In-depth analysis of the genetic framework of β-cells might identify new transcription factors that drive functional maturation of β-cells. Indeed, several groups reported the expression of *SIX2* and *SIX3* in human β-cells [41,45,48,51]. These two transcription factors have recently been shown to elevate insulin content and insulin secretion in juvenile, immature β-cells suggesting a crucial role of *SIX2* and *SIX3* in β-cell maturation [52]. Importantly, *Six2* and *Six3* seem not to be expressed in adult mouse β-cells. Altogether, these findings call for caution when extrapolating findings from mice to humans and highlight the importance of studying human pancreatic cell types as well as indicate the requirement of cell-type specific mapping of genes.

So far, our understanding about the transcriptional program and function of pancreatic δ- and γ/PP-cells is limited due to their low

abundance. Only recently, DiGrucchio et al. reported that murine δ-cells express several receptors, which they partially have in common with β-cells, such as *glucagon-like peptide 1 receptor* (*Glp1r*), the *alpha 2 adrenergic receptor* (*Adra2a*), and the *glucagon receptor* (*Gcgr*), whereas the expression of *somatostatin receptor* (*Sstr1*) and *ghrelin receptor* (*Ghsr*) seems to be restricted to δ-cells [53]. Ghrelin has a central role in energy and glucose metabolism and a well-known insulinostatic action [54], which can now be explained by its direct effect on somatostatin-releasing δ-cells. Several single-cell RNA sequencing studies confirmed the expression of *GHSR* in human δ-cells and additionally identified the leptin receptor (*LEPR*) as a δ-cell specific receptor [41,45,48,51]. Leptin is an adipose tissue-derived hormone that plays an important role in the maintenance of body weight and glucose homeostasis [55]. Until now, the peripheral target cell of leptin action was controversial. Several studies in mice have suggested a direct effect of leptin on β-cells. It has been shown that leptin suppressed insulin release from insulinoma cells and pancreas-specific leptin receptor knock-out mice exhibited improved glucose tolerance, increased early-phase insulin secretion and increased β-cell size [56,57]. However, leptin receptor deletion in insulin- or proglucagon-expressing cells had only a minor impact on glucose homeostasis [58]. Thus, the δ-cell-specific expression of the leptin receptor would explain the conflicting results obtained by a pancreas-specific (including δ-cells) versus β/α-cell-specific leptin-receptor deletion. Interestingly, several diabetes risk genes such as *PDX1*, *HADH*, and *UCP2* are expressed by β- and δ-cells, thus implicating the δ-cell type in islet dysfunction in rare and common forms of diabetes [51]. Taken together, these new data strongly suggest an important role of δ-cells in maintaining β-cell function and systemic regulation of appetite, food intake, and energy balance.

Little is known about the function of the rare γ/PP-cells. Human γ/PP-cells have now been reported to express high levels of the transcription factors *MEIS2*, *ETV1*, *ID4*, and the serotonergic transcription factor *FEV/PET1* besides *TPH1*, *SERTM1*, *SPOCK1*, *ABCC9*, and *SLITRK6*, suggesting similarity of γ/PP-cells with neuronal cells [41,44,45,51]. To summarize, recent single-cell studies reveal the transcriptional program of rare islet cell-types and species-specific differences in gene expression important for the interpretation of conditional knock-out studies in mice and for consideration when designing new therapies for diabetes (Figure 3).

4. APPROACHES OF ENDOGENOUS β-CELL REGENERATION

4.1. Targeting β-cell subpopulations

Endogenous regenerative therapy aims to stimulate subset(s) of cells with the potential to compensate for the lost functional β-cell mass. A tempting source in this regard is the remaining (dedifferentiated) β-cells that survive disease for long periods. It has been reported that in T1D patients a small amount of functional β-cells remain for many years and escape immune attack [59–61]. Similarly, not all β-cells undergo cell death in T2D patients. A fraction of cells regress to a more immature state, creating a pool of possible precursor cells that can potentially be re-differentiated [3,62,63]. In addition, in an early stage of disease, there are still functional β-cells, which may be expanded by inducing their proliferation. The molecular differences that cause cells to adopt distinct fates and make them survive, die, or proliferate are still elusive. Thus, to directly target specific β-cell subpopulations and trigger their proliferation and/or functional maturation, it is crucial to decipher β-cell heterogeneity and to identify the underlying molecular mechanism driving it. It is long known that β-cells are a heterogeneous population of cells that differ in morphology, glucose responsiveness,

insulin secretion, proliferative capacity and maturation state [11,64–67]. Regardless of these extensive studies of phenotypic and functional β -cell heterogeneity first described over 50 years ago, heterogeneity has only recently moved into the spotlight of regenerative diabetes research [11,68,69]. Besides pathological dedifferentiation, heterogeneity may be caused by differences in the microenvironment, islet architecture, and distinct origins of β -cells. Markers that are differentially expressed in β -cells in homeostasis include insulin [67,70], the transcription factor Pdx1 [71], the membrane transporter glucose transporter Glut2 [72], and signaling components such as WNT antagonist DKK3 [73] among many others [11]. β -cell heterogeneity has been mostly studied in rodents. The detection of β -cell heterogeneity within human pancreatic cell populations remains currently a challenging task due to large donor-to-donor variability [41,74], specific loss of β -cells during the experimental procedure [41], and a requirement to analyze a large number of cells. Nonetheless, recent single-cell studies confirm that murine and human β -cells differ in their proliferative capacity, insulin secretion, and response to diabetogenic cues as discussed below (Figure 4).

4.1.1. Triggering β -cell proliferation

Pancreatic islets possess the functional flexibility to adapt rapidly to environmental changes by β -cell mass expansion and enhanced insulin secretion. Exploiting natural proliferation cues to increase cell number in disease, therefore, is evident. β -cell proliferation peaks during the early postnatal period, in which β -cell mass is determined. Qiu et al. showed that 25% of the β -cells are proliferative on postnatal day (P) 3 in mice based on the expression of cell cycle-related genes. However, from P9 onwards proliferation decreases rapidly and the adult β -cell turnover is minimal (below 1% in mice) but stable [46,74,75]. Understanding the molecular mechanisms that drive the early postnatal β -cell proliferation boost and induce cell cycle arrest of most β -cells thereafter might be helpful for therapeutic approaches. Two recent single-cell RNA-sequencing studies aimed to reconstruct the developmental trajectory of pancreatic β -cells to gain insight into the regulation of postnatal proliferation and maturation. Zeng et al. used the *mIns1-H2B-mCherry* reporter mouse line to isolate β -cells whereas Qiu et al. made use of the *Ins1-RFP* and *Gcg-Cre; Rosa-RFP* reporter mouse lines to isolate β -cells and α -cells, respectively [46,76]. In both studies, the maturation trajectory was reconstructed by ordering β -cells isolated from multiple postnatal time points based on their transcriptional similarity. Qiu et al. reported 664 genes and 448 genes to be dynamically regulated during the β -cell maturation and α -cell maturation processes, respectively. Interestingly, their results suggest that β -cells mature primarily through the upregulation of genes. In contrast, α -cells seem to mature through downregulation of genes expressed in immature α -cells [46]. Furthermore, pseudotemporal cell ordering revealed the signatures of immature, proliferative β -cells and associated expression changes of genes regulating amino acid uptake and metabolism, mitochondrial respiration, and reactive oxygen species (ROS) production with postnatal β -cell development. Precisely, the results of the Sander group suggest that amino acid deprivation due to downregulation of amino acid transporter genes, as well as reduced ROS level and downregulation of *serum response factor* (*Srf*) and its target genes during β -cell maturation might contribute to the postnatal decline in β -cell proliferation [76]. Other pathways that are regulated in pseudotime and implicated in proliferation are MAPK and PDGF signaling [76]. In addition, the transcription factor *Foxm1* and several members of the pro-proliferative *E2F* transcription factor family and their targets are highly expressed in postnatal proliferative β -cells [46,76]. In that

respect, it is also worth mentioning that cell cycle exit/proliferation seems to be differently regulated in immature and mature α -/ β -cells as the cell cycle inhibitors p57 and p18 are highly expressed in immature and mature islet cells, respectively [46]. Thus, targeting the ROS/SRF/MAPK/PDGF pathways and amino acid availability might reactivate and stimulate proliferation in adult human β -cells.

As a boost of β -cell proliferation is observed under high metabolic demand, such as pregnancy or obesity, it would be interesting to investigate if pathways implicated in the regulation of early postnatal β -cell proliferation are reactivated under these conditions [77]. However, increased β -cell proliferation upon metabolic demand shows that at least a subset of adult β -cells maintains the ability to replicate.

Unlike tissues with rapid cell turnover, such as skin, blood, or gut, the presence of stem cells in the pancreas is controversial [10]. Even though neogenesis of ductal facultative stem cells residing outside the islets may contribute to formation of new β -cells [78,79], lineage-tracing studies indicate that self-replication of pre-existing β -cells is the major source of new β -cells *in vivo* in homeostasis and upon injury [80]. Others reported the existence of a small (< 1%) adult pancreatic multipotent progenitor (PMP) population within both mouse and human islets. The described PMP cells are insulin-positive and show high proliferative capacity, can give rise to all endocrine cell types *in vitro*, and may contribute to β -cell compensation under stress and hyperglycemia [72,81,82]. The presence of PMPs would have major implications for regenerative therapy; however, due to the very low cell turnover in the islet the concept needs verification. Identification of marker genes for the isolation of PMPs as well as stem cell assays to test potency and the ability of self-renewal are warranted [10].

To efficiently stimulate β -cell replication, it is important to delineate heterogeneities in the replicative potential of cells. The presence of adult, proliferative human β -cells in homeostasis is supported by a single-cell mass cytometry study, in which islets of 20 human donors covering ages from birth to adulthood were profiled [74]. In accordance with previous studies, cell percentage and proliferation of all major endocrine cells was highest neonatally and declined after childhood with some basal proliferation remaining in adults. Moreover, hierarchical clustering revealed three, distinct β -cell states. Proliferative cells segregated into two of these subgroups. The cluster containing most of the Ki67-positive β -cells also showed higher levels of the proliferation marker Ki67 and an upregulation of signaling components involved in β -cell proliferation (PDGFRA, pERK1/2, pSTAT3, and pSTAT5) [83]. The surface markers CD44 and CD49F were identified among the proteins highest expressed in proliferative β -cells, which have been associated with pancreatic progenitor cells and pancreatic cancer-initiating cells [84,85]. Strikingly, the number of proliferative cells is decreased in T2D donors [74]. Nevertheless, treatment with the drug harmine, which has been previously demonstrated to enhance human β -cell proliferation [83,86,87], had similar effects on various endocrine cells from T2D and healthy donors indicating that even in an impaired metabolic state endocrine mass can be expanded.

So far, single-cell RNA sequencing captured proliferative β -cells only from early postnatal stages [46,76]. This is possibly due to the very low replication rate inherent to adult β -cells [74] and high sensitivity of proliferative β -cells to flow cytometry, on which many single-cell technologies rely [41]. In addition, most studies to date analyzed a limited number of cells or lacked an in-depth analysis of β -cells, and, therefore, rare cell types might have been missed. Still, several single-cell RNA sequencing studies found indications for the presence of proliferative β -cell subpopulations in adult islets. The Sandberg group identified five clusters within the β -cell population, of which three showed elevated expression of *inhibition of differentiation* (*ID*) genes

PHENOTYPE		mature	immature	proliferative	other	genes/ proteins
β-cells	GPR120 ⁴¹	■	■	-		<i>GPR120</i>
	RBP4 ^{41,45,48,61}	■	■	-	downregulated in resistant cells in T1D	<i>RBP4</i> , β-cell markers (e.g. <i>UCN3</i> , <i>MAFA</i> , <i>CHGA</i>)
	ER stress ^{45,48,76}	■	■	91	absent in T2D, upregulated in early postnatal β-cells	<i>HERPUD1</i> , <i>DDIT3/Ddit3</i> , <i>TRIB3</i> , <i>HSPA5</i> , <i>PP1R15A</i> , <i>LURAP1L</i> , <i>ATF3/Atf3</i> , <i>FTH1P3</i> , <i>SOSTM1</i> , <i>FTH1</i> , <i>FTL</i> , <i>SRXN1</i> , <i>Atf4</i> , <i>C/EBP</i>
	ID genes ^{41,76}	■	88,89,90	88,89,90	absent in T2D, upregulated in early postnatal β-cells	<i>ID1</i> , <i>ID3</i>
	CD44/CD49F ⁷⁴	■	-	■	absent in T2D	<i>Ki67</i> , <i>PDGFRA</i> , <i>pERK1/2</i> , <i>pSTAT3</i> , <i>pSTAT5</i>
	Ucn3 ¹¹⁰	■	■	-	intermediate stage in α- to β-cell/β- to α-cell conversion, neogenic niche at islet edge, no glucose sensing	<i>Ucn3</i> , <i>G6pc2</i> , <i>Eroib</i> , <i>Glut2</i>
α-cells	SHH ^{41,63}	-	-	■		<i>Ki67</i> , <i>GLI</i> factors, <i>DYRK1A</i> , <i>GSK3β</i> , <i>STMN1</i>
	STMN1 ^{41,116}	-	-	■		<i>STMN1</i> , <i>SOX9</i>
acinar cells	REG3A ⁴⁸	■	48,129	129,130	spatial patches	<i>REG3A</i>
	MHC class II ⁴¹	■	-	-	inflammatory	<i>CD74</i> , <i>HLA-DMA</i> , <i>HLA-DRA</i> , and <i>HLA-DRB1</i> , <i>CXCL1</i> , <i>CXCL6</i> , <i>CCL2</i> , <i>CCL20</i> , and <i>CX3CL1</i> , <i>IL17C</i> , <i>IL18</i> , <i>STAT1</i> , <i>NFKBIA</i> , <i>NFKBIZ</i> , <i>HIF1A</i> , <i>SOX4</i> , <i>ONECUT2</i>
duct cells	CEACAM6 ¹⁴¹	-	■	-	progenitor-like linked to α-, δ-cells	<i>CEACAM6</i>
	FTH1/FTL ¹⁴¹	-	■	-	progenitor-like linked to β-, γ/PP-, acinar cells co-expression with insulin	<i>FTH1</i> , <i>FTL</i>
	MUC1 ⁴⁵	■	-	-	mucous secretion, centroacinar	<i>MUC1</i> , <i>MUC20</i> , <i>TFF1</i> , <i>TFF2</i> , <i>PLAT</i>
	CFTR ⁴⁵	■	-	-	ion transport, terminal duct	<i>CFTR</i> , <i>CD44</i>

■ upregulation/high expression
 ■ downregulation/low expression
 ■ no expression
 xxx link to phenotype based on literature
 - no link in discussed single-cell publications

Figure 4: New heterogeneities in pancreatic cell types. Table summarizing the new concepts and markers of cellular heterogeneity in the pancreas that are discussed in the text.

[41]. ID transcription factors have been shown to be involved in the regulation of cell proliferation and inhibition of differentiation in several cell types [88]. Specifically, upregulation of IDs has also been associated with cancerous pancreatic tissue, expansion of ductal progenitors, and inhibition of endocrine cell differentiation [89,90]. Interestingly, *Id1* and *Id3* are also highly expressed in early postnatal β-cells, in which they are implicated in the regulation of ROS, an important driver of postnatal β-cell proliferation [76]. Further, two groups independently detected a human β-cell subpopulation expressing genes that function in the unfolded protein response (UPR), also known as endoplasmic reticulum (ER) stress [45,48]. Recent work has linked high insulin demand and β-cell proliferation to mild ER

stress. Sharma et al. suggest that β-cells sense insulin demand via the UPR mechanism, which triggers proliferation [91]. Importantly, the set of genes upregulated in this novel subtype overlap between the studies (e.g. *HERPUD1*, *DDIT3*, *TRIB3*, *PP1R15A*, *LURAP1L*, *ATF3*), which indicates their biological significance. In agreement, Zeng et al. observe a downregulation of ER stress related genes (e.g. *Atf4*, *C/EBP*, *Ddit3*) concomitant with downregulation of genes associated with proliferation during postnatal β-cell maturation in mice, further supporting a role of mild ER stress in the control of the proliferative response [76]. Together, these studies hint at proliferative β-cell subpopulations owing the potential to restore part of the β-cell mass and at underlying pathways including UPR/ER stress pathway, SRF- and ROS-mediated

signaling, as well as PDGF and JAK/STAT signaling, but, clearly, more detailed knowledge is required to target β -cell self-replication for therapeutic purposes.

4.1.2. Triggering β -cell maturation

The most stringent prerequisite to effectively restore metabolic homeostasis is to achieve full functionality and maturity of the newly generated β -cells. As part of naturally reversible events and aging, β -cells are expected to undergo a spectrum of changes in adulthood equivalent to varying differentiation states [92]. A mature β -cell phenotype is generally associated with high levels of Insulin (Ins) and expression of β -cell-specific glucose transporter 2 (Glut2) as well as of the transcription factors MafA and Nkx6.1 among others. In contrast, immature cells in mice are described by elevated expression of genes involved in early β -cell development (*MafB*, *Pax4*, *Pax6*, and, in extreme cases, *Ngn3*) and decreased insulin secretion, in brief a loss of key maturation factors needed for optimal function [3,93]. The identification of markers and a detailed characterization of the different stages would be of great benefit, since hyperglycemia in diabetes is thought to cause a fraction of β -cells to move toward a more primitive state, a process often termed dedifferentiation [3,92]. Recent work suggests that even in T1D a subpopulation expressing immature features persists long-term immune attack (for more detail see section 4.1.3) [61].

The presence of immature β -cells in adult islets has been reported in mice and human. Szabat et al. detected two stable subpopulations of PDX1 positive β -cells with distinct insulin levels [71]. 25% of human and mouse β -cells were PDX1⁺/Insulin^{low} and displayed an immature expression profile together with an increased replication rate and diminished insulin secretion. Moreover, a significant fraction of these cells transition into a more mature state in culture. The recent findings from our lab further confirm the presence of immature or pre- β -cells and link active WNT/planar cell polarity (PCP) signaling to β -cell maturation [69]. We showed that Flattop (Ftpt), a downstream effector and reporter of the WNT/PCP pathway is heterogeneously expressed among pancreatic endocrine cells and subdivides β -cells in mice into Ftpt Venus reporter (FVR)-negative proliferative cells (20%) and FVR-positive metabolically active cells (80%) [69,94]. Intriguingly, in-depth analysis revealed that FVR-negative β -cells show characteristics similar to the proposed PMPs (Insulin⁺, Glut2^{low}), which suggests that proliferative potential varies among β -cells in the adult islet and indicates that pre- β -cells might be enriched in the FVR-negative population. Moreover, a genome-wide transcript profiling array of FVR-positive and FVR-negative endocrine populations showed differential expression of WNT and MAPK signaling components, β -cell maturation markers, genes associated with G-protein coupled receptor (GPCR), hormones, proliferation markers, and glycolysis enzymes, which suggests that β -cell subpopulations indeed can be selectively targeted [69]. Interestingly, WNT and MAPK signaling components are highly expressed in both immature, postnatal β -cells, and FVR-negative endocrine cells [46,69]. However, Qui et al. did not detect differences in the transcriptional profile of Ftpt mRNA-positive and Ftpt mRNA-negative β -cells [46]. The reason is due to the transient expression of the Ftpt mRNA during WNT/PCP acquisition [[69,94] and Böttcher et al. in preparation]. As already speculated by the authors, the WNT/PCP pathway acts preferentially at the post-transcriptional level to establish planar polarization, which is accompanied by the acquisition of distinct physiological features. Therefore, while differences at the transcriptional level are minor, post-translational WNT/PCP signaling is essential to trigger a mature β -cell phenotype. These studies emphasize that the analysis of proteins and post-translational

modifications in single cells is warranted to reveal in-depth knowledge of physiology and disease.

Together, these results demonstrate that β -cells pass through different maturation states in adult islets. This implies that promotion of both replication and maturation is required to obtain functional, insulin-producing β -cells. The FVR-negative cells described above show a combined proliferative and immature phenotype and eventually mature into insulin-secreting cells, which is triggered by active WNT/PCP signaling [69]. Similarly, a number of recent single-cell studies reported on heterogeneities in expression of genes involved in insulin regulation and β -cell development. Apart from differential expression of *ID* genes (see above) the Sandberg group detected two clusters with high levels of *serum retinol-binding protein (RBP4)* and *GPR120* (also known as *FFAR4*) [41]. The adipokine RBP4 promotes insulin resistance and is increased in obese and T2D individuals [95], whereas engagement of GPR120 induces insulin secretion [96]. Strikingly, Baron et al. also identified a β -cell state with elevated expression of *RBP4* concomitant with the expression of marker genes of mature β -cells [45]. Further, Muraro et al. described a β -cell subpopulation expressing ER stress genes indicative of less mature cells [48]. Looking at the expression fold change in the published data of these cells shows a slight decrease of *RBP4*, which is, however, not significant. Interestingly, *RBP4* expression is also downregulated in a β -cell subpopulation resistant to the immune response associated with T1D [61]. Together, this suggests a role of RBP4 also in homeostatic regulation of insulin secretion, which is potentially impaired in diabetes and contributes to dysfunction and disease. While *RBP4* shows different expression levels, *GPR120* seems to be completely absent in a fraction of cells [41]. Given its function in insulin release, GPR120 is an interesting candidate marker for functional and mature β -cells. In this context, it is important to note that cells might not clearly separate into distinct maturation states due to the continuous nature of the maturation process and the presence of transitioning cells. Indeed, Baron et al. describe rather gradual changes in gene expression over the whole β -cell population, instead of clear on/off states [45]. Taken together, single-cell analysis identified new potential markers of mature β -cells. However, to molecularly dissect β -cell heterogeneity and to better understand and drive β -cell maturation we need more information on regulatory elements such as transcription factors and signaling molecules, which are low in expression but might have a strong effect on cell fate and state (Figure 4).

4.1.3. β -Cell heterogeneity in the pathogenesis of diabetes

The available data clearly support the presence of β -cell heterogeneity in adult islets; its role in the pathogenesis of diabetes mellitus, however, is not fully understood. Single-cell studies considerably extended the list of genes with aberrant expression in β -cells of diabetic patients. Most of the recent single-cell studies, however, failed to detect β -cell subpopulations and thus ignore the evidently present heterogeneity [42,51,63]. Distinct β -cell subpopulations might respond differently to diabetic stressors. Likewise, not all cells are equally susceptible to therapeutic interventions. In addition, metabolic stress itself might contribute to heterogeneity, as novel dysfunctional β -cell subtypes can arise or mature cells dedifferentiate to a more immature state. Indeed, Baron et al. found indication of a disappearing β -cell subtype under hyperglycemic conditions [45]. They detected downregulation of ER stress marker genes such as *Herpud1* in β -cells in the diabetic state that were associated to a β -cell subpopulation in healthy pancreatic islets [45]. Accordingly, Dorell et al. describe two surface markers, ST8SIA1 and CD9, that discriminate four β -cell subpopulations in the human adult islets [68]. All four populations

expressed common β -cell markers but displayed differences in insulin secretion rates and in their gene expression profiles. Importantly, the subtype distribution was altered in T2D islets highlighting the relevance of the described β -cell heterogeneity and its implication in disease. In line with these results, three distinct β -cell groups were identified that shift in number in T2D or with age and BMI [74]. All three studies could not clarify whether altered partitions of cells were a cause or consequence of β -cell dysfunction. In addition, it remains unclear whether these subtypes have a temporal relationship or are independent lineages and whether distorted proportions result from the selective loss of a β -cell subtype and/or from cellular transitions. More detailed molecular profiles of these β -cell subgroups are required to determine their biological significance, function, ontogeny, and implication in disease. A challenge in this regard is the high donor-to-donor variation that was detected by multiple single-cell studies [41,45,74]. Conclusions on islet cell composition in homeostasis and disease, therefore, should only be drawn if they are robust over multiple donors.

Records of subpopulation-specific expression profiles also would help to solve the debate on the contribution of partial dedifferentiation to the reduction of functional β -cell mass in T2D. β -cell dedifferentiation is characterized by diminished expression of β -cell specific transcription factors (e.g. *PDX1*, *NKX6.1*, *MAFA*), reactivation of developmental genes (e.g. *Ngn3*) and, surprisingly, pluripotency genes (e.g. *Oct4*, *Nanog*, *L-myc*), as well as decreased insulin production [3,62,92,97] and expression of disallowed genes that are silenced in healthy β -cells including *lactate dehydrogenase A (Ldha)*, the *monocarboxylate carrier (Mct1)*, as well as *glucose-6-phosphatase (G6pc)*, and *hexokinase I (Hk1)* [98,99]. Recent studies demonstrated that acquiring and maintaining β -cell identity and function also requires sustained repression of other endocrine gene programs and, thus, give an explanation on how adult β -cells can partially dedifferentiate or become reprogrammed into other islet endocrine cells [27,28,100]. Indeed, Wang et al. found evidence of dedifferentiated cells in diabetic islets when comparing their transcriptional profiles to cells of healthy patients [63]. β -cells of adult T2D individuals exhibit transcriptional patterns of juvenile endocrine cells indicating partial regression to an immature state characterized by a less well-defined α - and β -cell gene signature and expression of *CDKN2B*, *BARD1*, *JUNB*, and *PRKD1*. Segerstolpe et al. reported significantly lower *INS* mRNA levels in T2D β -cells, a feature of dedifferentiated β -cells [41]. However, the authors of both studies did not comment on the expression of *NGN3*, *FOXO1*, or other factors associated with β -cell dedifferentiation.

In a recent study, a novel murine β -cell subpopulation was described that develops during progression of T1D in response to immune cell activity and persists for a long time [61]. These cells resist immune assault, exhibit increased proliferation rates, and acquire stem-like and immature features while the expression of mature β -cell marker genes, insulin content, and diabetes antigens is decreased. A similar, less-differentiated β -cell subpopulation appeared when human islets were co-cultured with allogeneic lymphoid cells. These changes in gene expression likely account for the long-term survival of a few β -cells in T1D patients [61]. Together, these studies indicate that a group of β -cells adapt to metabolic and immune stressors in T2D and T1D, respectively, by regression to an immature state. To what degree dedifferentiation occurs in diabetes and whether β -cells revert to a multipotent precursor or a novel, reversible dedifferentiated state will have to be established [62,101]. Nevertheless, dedifferentiation clearly contributes to the etiology of diabetes together with β -cell death and creates a pool of cells that can possibly be triggered to redifferentiate

and reestablish islet function. Since β -cell dedifferentiation is associated with and possibly caused by hyperglycemia, reconstitution of normoglycemia might restore a normal mature β -cell phenotype [92]. In summary, β -cell subpopulations show varying responses to metabolic stress; thus, heterogeneity needs to be considered to gain an understanding of the pathomechanisms of diabetes and to identify therapeutic targets (Figure 4).

4.2. Intra-islet cell transdifferentiation

Recent findings in mice have revealed unexpected plasticity and potential of intra-islet (e.g. α -cell) and extra-islet (acinar and ductal) cells to transdifferentiate and produce insulin. Transdifferentiation is defined as a process whereby a differentiated cell is converted into another type of cell either directly via a double hormone-positive intermediate cell or through a dedifferentiated state. Examples for both forms exist in the pancreas and are reviewed in Puri et al. [12].

4.2.1. α - to β -cell conversion

α -cells secrete the hormone glucagon, which induces glycogenolysis to elevate blood glucose levels. Importantly, α -cells are more resistant to metabolic stress than β -cells, and their number does not significantly change in T1D and T2D patients [41,51,63,102]. Considering the close developmental and physiological relationship between α - and β -cells as well as the big overlap of their transcriptome, α -cell transdifferentiation draws much attention as a source for β -cell regeneration. Transdifferentiation of α -cells into β -cells has been observed by several groups in different experimental settings [47,103–109]. Single gene manipulations suffice to induce α -cell conversion towards the β -cell fate, as first shown by Collombat et al. through ectopic overexpression of *Pax4* [103]. Interestingly, besides forced expression of key transcription factors, β -cell depletion after puberty drives the conversion of remaining α -cells albeit by a different mechanism; nevertheless indicating a natural regeneration mechanism that could be triggered in diabetic conditions [104]. The recent identification of urocortin3-negative, insulin-expressing β -cells (1–2% of all β -cells) that are transcriptionally (lack expression of e.g. *G6pc2*, *Eroib*, and *Glut2*) and functionally (do not sense glucose) immature suggests a naturally occurring α - into β -cell conversion as part of islet homeostasis [109]. Lineage-tracing studies revealed that these cells represent an intermediate stage in both the transdifferentiation of α -cells to mature β -cells as well as the inverse transition from β -cells to functional α -cells. Interestingly, transdifferentiation takes place within a specialized neogenic niche at the periphery of healthy islets, which might exist in humans, at least at a younger age, as well [109]. Other studies also reported on ‘intermediate’ cells expressing both α - and β -cell markers in human islets [70]. The transcriptional profile of double hormone-positive cells could give insights into the mechanisms underlying α - to β -cell conversion and unravel driving factors. Indeed, such rare double hormone-positive cells were captured by single-cell RNA sequencing studies but not further commented on or excluded as doublet cells [41,63].

The relative ease with which α -cells can be transdifferentiated into β -cells may be partly explained by their plastic epigenomic state. α -cells have hundreds of bivalent activating and repressing histone marks on developmental genes, strikingly similar to the histone modification map of human embryonic stem cells, indicating an undifferentiated multipotent epigenomic state [105]. Indeed, inhibition of histone methyltransferases resulted in the appearance of bi-hormonal, insulin-, and glucagon-positive cells. Analysis of open chromatin in α - and β -cells using the assay for transposase-accessible chromatin with th-

roughput sequencing (ATAC-Seq) confirmed a bivalent chromatin state in α -cells [47]. In addition, α -cells have more open chromatin regions compared to β -cells, of which many are associated with β -cell signature genes. Interestingly, simultaneous inactivation of the α -cell regulator *Arx* and DNA methyltransferase 1 (*Dnmt1*) is sufficient to promote rapid conversion of α -cells into β -like cells capable of insulin production and secretion [110], which substantiates that the conversion process involves epigenetic changes in addition to modulation of cell-type specific master regulators. Single-cell RNA sequencing and functional evaluation revealed strikingly little difference between converted and native β -cells but uncovered cells retaining α -cell character suggesting that not all α -cells are equally susceptible to reprogramming [110].

Two recently published studies report that stimulation of γ -aminobutyric acid (GABA) signaling can induce β -cell regeneration and reverse severe diabetes in mice [106,107]. Ben-Othman et al. describe that long-term administration of GABA induces α -cell mediated β -like cell neogenesis *in vivo* [106]. Precisely, GABA triggers conversion of α -cells to functional β -like cells through downregulation of *Arx*, the transcriptional repressor of *Pax4*. This, in turn, stimulates α -cell regeneration mechanisms involving the reactivation of *Ngn3*-controlled endocrine developmental processes and increases proliferation of duct-lining progenitor cells, which gives rise to new α -cells. The newly generated α -cells are subsequently converted into β -cells upon prolonged GABA exposure. GABA treatment results in increased islet number and size, and β -cell mass could be repeatedly replaced in chemically induced diabetes. Most important, human islets responded similarly to GABA, indicating that the findings in mice indeed might be translatable to humans. In a complementary study, Li et al. found that the antimalarial drug artemether exerts similar effects by binding to gephyrin, a protein associated with the GABA receptor complex [107]. Together these studies suggest a therapeutic potential of GABA pathways, but, clearly, further research is needed to confirm these results and to clarify how GABA acts in diabetic human islets. Although, α -cell mass and metabolism seem not to be strongly affected by diabetes, gene expression is clearly changed. Up to 200 differentially expressed genes between healthy and T2D islets were identified by single-cell transcriptomics, of which approximately 35% overlap between different studies [41,42,51,63]. This suggests altered α -cell states, which might influence GABA treatment efficiency. Thus, whether adaptive neogenesis of α -cells induced by GABA is possible in humans remains to be shown.

Besides α -cell replacement from exocrine tissue, a reserve α -cell pool that converts into β -cells upon metabolic stress could also be established by triggering α -cell proliferation. Strikingly, of all major endocrine cell types, α -cells show the highest basal proliferation rate as well as the most robust mitotic response to the mitogen harmine across healthy and T2D donors [74]. Concurrently with these findings, single-cell transcriptomics identified and enabled transcriptional profiling of rare proliferating α -cells [41,46,63]. The Kaestner group identified a single proliferating α -cell out of 190 annotated α -cells, which showed high expression of the proliferation marker *Ki67*, activation of cell cycle pathways and inhibition of cell cycle checkpoint control genes [63] (Figure 4). In addition, both *DYRK1A* and *GSK3 β* were downregulated, which is consistent with their suggested role as inhibitors of endocrine cell replication [86,111]. Common targets of both proteins are the GLI transcription factors, which implicates modulation of the Sonic Hedgehog (SHH) signaling pathway in replicating α -cells [112–114]. Segerstolpe et al. also detected a small population of proliferative α -cells with increased expression of

proliferation-associated genes and slightly reduced levels of several α -cell specific markers [41]. This proliferating subtype was further distinguished by 439 significantly differentially expressed genes. Interestingly, when looking more closely at the list of the differential gene expression analysis, we see that the transcriptional regulator of the SHH pathway *GLI2* is enriched, however not significantly. Moreover, the expression of *Stathmin (STMN1)*, which is associated with dividing acinar cells (see below) and progenitor cells in other organs, is significantly increased in proliferative α -cells [115]. Taken together, the high-dimensional single-cell profiles provide information on activated and repressed pathways in replicating cells and potential targets to trigger α -cell proliferation.

The accumulating evidence supports the model that α -cells represent a natural source of new β -cells. α - to β -cell transdifferentiation can be induced by rather simple genetic and epigenetic manipulations or drug treatment and to some extent occurs spontaneously in homeostasis and upon β -cell depletion or in T1D. Together with the higher rate of proliferation, the bivalent histone modifications at loci of developmental genes and open-chromatin regions in β -cell genes, this makes α -cells a tempting target for future clinical applications in T1D and T2D and imply that α -cells might have an important function besides glucagon secretion.

4.3. Extra-islet cell conversion

4.3.1. Acinar to β -cell conversion

Acinar cells are the major constituent of the adult pancreatic tissue and responsible for the production and secretion of digestive enzymes. Owing to their abundance and origin from common pancreatic progenitor cells, acinar cells represent an appealing pool for β -cell replacement. Several studies reported successful conversion of acinar cells into endocrine cells *in vivo* and *in vitro* either via a pancreatic progenitor state or by direct lineage switching [12]. Acinar-to- β -cell reprogramming can be stimulated by ectopic expression of specific transcription factors [116,117], administration of signaling and growth factors [118–120] and co-transplantation with fetal pancreatic cells [121–123]. The Melton group was the first to show successful *in situ* formation of cells with β -cell like function and morphology in mice by the expression of β -cell transcription factors (*Pdx1*, *Ngn3*, *MafA*) in acinar cells [116]. Importantly, human acinar cells also can be induced to transdifferentiate into insulin-producing β -cells [124]. The contribution of acinar cells to β -cell regeneration *in vivo*, for example after injury, remains unclear. Moreover, inconsistencies exist as to whether acinar cells assume a β -cell state through transdifferentiation or dedifferentiation to a facultative progenitor or both [125]. Genetic-lineage tracing experiments using acinar specific promoters could not yet clearly identify the origin of new β -cells after triggering cell conversion [119,120]. To harness acinar cell reprogramming towards β -cell production in diabetic patients, we need a better understanding of the factors that control the lineage conversion process and clarify if and to what extent it is a natural process in regeneration. To this end, high-dimensional analyses at the single-cell level can give important insights. Until now, the existence of possible heterogeneity in acinar cells that might influence reprogramming efficiency has been mostly neglected in conversion protocols. Indeed, distinct acinar subclusters have been identified in single-cell studies [41,48]. In a very recent study, Wollny et al. found a progenitor-like acinar subpopulation in rodents with the capacity for long term self-renewal in homeostasis by lineage-tracing and organoid-formation assays [115]. Single-cell RNA sequencing of acinar cells and immunohistochemistry supported these

results and revealed a small subpopulation of dividing cells characterized by high expression of STMN1 (Figure 4). Stathmin plays an important role in the regulation of the cytoskeleton by destabilizing microtubules and has been identified as a marker for progenitor cells in other organs [126]. A subset of the STMN1-positive cells also expressed the transcription factor Sox9 that has been associated with exocrine progenitor cells of the pancreas [127]. Strikingly, the number of STMN1-positive acinar cells is increased upon injury, suggesting transient acquisition of proliferative capacity by normally non-proliferative cells. In line with these results, Segerstolpe et al. found a small subset of acinar cells (~4%) expressing proliferation markers [41]. They did not investigate these cells in detail, and it is not known whether they represent the proliferating acinar cells described by Wollny et al. [115]. A progenitor-like exocrine subtype could be a target for endogenous β -cell regeneration, and it would be interesting to see if the transcriptional profile of the proliferating acinar cells resembles a cell state of the acinar- β -cell reprogramming. Other groups also reported on acinar cell heterogeneity in single-cell transcriptomics data. Muraro et al. described differential expression of a member of the regenerating (Reg) protein family, REG3A, among acinar cells [48]. Reg proteins, first discovered in pancreatic inflammation and islet regeneration, exert anti-inflammatory, anti-apoptotic, and mitogenic effects in various physiological and disease contexts and are involved in differentiation and proliferation of various cell types [128]. REG3A in particular has been suggested to be involved in tumorigenesis of pancreatic cells by regulation of key genes and pathways implicated in cell growth [129]. Strikingly, REG3A-positive acinar cells formed cell patches close to the islets and showed lower expression levels of the acinar cell marker PRSS1 and key acinar genes encoding secretory enzymes (*CEL*, *CELA3A*, *CELA3B*, *AMY*). The reduced expression of functional acinar genes but increased level of proliferative signaling might suggest a more immature cell state potentially harboring the actively dividing acinar cells. More detailed analyses are required to confirm the existence of these cell subtypes and fully characterize them, to draw connections between them, and to investigate their function and plasticity. Proliferative or progenitor-like exocrine cells also may provide clues on regeneration mechanisms in endocrine cells since they are derived from a common progenitor. Furthermore, recent evidence indicates that acinar reprogramming efficiency is significantly reduced upon pancreatic inflammation or hyperglycemia but improved by inhibition of contact-mediated signaling [125,130–132]. This highlights the importance of investigating extrinsic factors that potentially hamper or enhance cell conversion *in vivo*. For example, acinar cells have been demonstrated to produce different inflammatory mediators as part of the first immune response to injury [133]. Consistently, Segerstolpe et al. detected a functionally distinct subpopulation that showed increased inflammatory markers in single-cell data [41]. If and how they influence reprogramming is an open question.

Taken together, acinar cells show plasticity *in vivo* and *in vitro* and could serve as a pool for β -cell regeneration. Importantly, results from rodents were translatable to human. However, the often contradictory experimental outcomes suggest that heterogeneity, contact-mediated lateral signals, the microenvironment, and physiological conditions also influence reprogramming and need to be understood to efficiently and reliably convert acinar into β -cells. Of note, genetic reprogramming and loss of acinar cell identity are critical early drivers of pancreatic ductal adenocarcinoma (PDA) formation, one of the deadliest malignancies [134,135]. Therefore, caution must be taken when manipulating acinar cell fate or triggering acinar cell proliferation.

4.3.2. Duct cell conversion into β -cells

The pancreatic duct drains the exocrine fluid into the duodenum and produces bicarbonate to regulate the luminal pH of acinar and duct cells [136]. Strikingly, ductal cells might have an important function beyond their physiological implication by providing a reserve pool of progenitor cells with the potential to give rise to endocrine cells, a process known as neogenesis. During the neonatal period in rodents, the β -cell pool further expands by β -cell replication as well as by neogenesis from ductal cells [79]. Neogenesis has also been reported to occur in certain experimental conditions such as ectopic expression of Pax4 in α -cells, upon which duct-lining cells sense glucagon-shortage and reactivate Ngn3 expression and associated developmental pathways to generate α -cells that eventually transdifferentiate into β -cells [103,137]. Xu et al. found that duct cells can give rise to new β -cells upon pancreatic duct ligation [138]. Furthermore, cultured murine adult pancreatic duct-like cells could be directly reprogrammed to insulin-producing β -like cells by adenoviral delivery of *Pdx1*, *Ngn3*, and *MafA* [139].

Whether adult duct lining cells also contribute to homeostatic renewal of β -cells is still under debate. Dor et al. concluded from their studies that replication and not neogenesis is the mechanism of β -cell expansion in adult mice [80]. In contrast, Seaberg et al. showed that besides islet cells, adult duct cells contain a population of pancreatic multipotent progenitors (PMP) that can give rise to all pancreatic lineages as well as neural lineages [81]. In agreement, Grün et al. identified two ductal cell clusters with a high multipotency score in the adult human pancreas using their newly developed StemID algorithm to detect potential stem cell populations within heterogeneous cell populations [140]. The inferred pancreatic lineage tree implies that distinct subtypes of ductal cells give rise to different endocrine subtypes and acinar cells. Precisely, they found that the cell cluster characterized by high *CEACAM6* expression, is linked to α - and δ -cells, whereas a cell cluster highly expressing the ferritin subunits *FTH1* and *FTL* is linked to β -, γ /PP- and acinar cells (Figure 4). Moreover, the authors also observed ductal as well as β -cells that co-express insulin and FTL, suggesting that ferritin-positive ductal cells differentiate into mature β -cells and implying that neogenesis contributes to homeostatic endocrine cell renewal. The ductal cell subpopulations reported by Baron et al. that are characterized by the expression of *CFTR* or *TFF1*, *TFF2*, and *MUC1*, most likely reflect their localization within the duct and form the terminal duct or connect to the acinus, respectively [45] (Figure 4). How these two duct cell populations relate to ductal PMPs or to the ductal subpopulations described by Grün et al. is not clear. Taken together, genetically manipulated mice show that neogenesis can be induced upon injury. Now, there is new evidence that ductal neogenesis not only occurs in the neonatal period and to some extent in homeostasis and upon injury in rodents but might indeed be part of homeostatic renewal of endocrine cells in humans [78]. Further work is necessary to identify the signals that mobilize ductal progenitors to provide a route to replenish β -cells *in situ*. In rodents, ductal cells convert either into β - or α -cells depending on the injury mode. To identify the signals and transcriptional program that specifically drive ductal into β -cell conversion, we need to study the behavior of ductal cells in different injury models at the single-cell level. Notably, the presence of ductal progenitor cell giving rise to β -cells could also explain part of the observed β -cell heterogeneity.

5. CONCLUSION

Here, we reviewed recent literature on single-cell analysis of pancreatic cell types and discussed the findings with respect to

endogenous β -cell regeneration routes. Single-cell genomics in the human pancreas is still at an early stage. Nevertheless, it has already provided new insights into the transcriptional program of pancreatic cells including those of rare cell types such as δ - and γ /PP cells. Expression of the leptin receptor and diabetes risk genes, such as *PDX1*, *HADH*, and *UCP2* in δ -cells for instance strongly suggests an important role of δ -cells in the maintenance of glucose homeostasis despite their low number and demands to study δ -cells in more detail [41,45,48,51].

One of the most promising strategies to restore β -cell mass is stimulation of β -cell replication and/or maturation. Single-cell transcriptomics data on postnatal, murine β -cells revealed the signature of immature β -cells and implicate ROS, ER stress, SRF, MAPK, TGF- β , WNT, and PDGF signaling in the regulation of postnatal β -cell proliferation and maturation [46,76]. Strikingly, PDGF and MAPK signaling as well as ER stress have also been associated with potentially proliferative and regenerative adult human β -cell subpopulations [45,48,74]. Thus, modulating these pathways could be a strategy for reactivating and promoting the expansion and maturation of residual β -cells in diabetic patients.

Reprogramming of intra-islet cells into β -cells is an alternative approach considered for β -cell regeneration. New data implicate a contribution of α - into β -cell conversion as well as ductal neogenesis to homeostatic β -cell renewal in mice [109,140]. Since severe β -cell depletion is known to trigger transdifferentiation of α -cells into β -cells in mice and α -cells are the most proliferative cell-type among the endocrine cells, employing α -cells might be a promising approach for β -cell regeneration [74,104]. Therefore, identifying the signals that drive α -cell proliferation and α -cell into β -cell conversion in homeostasis and upon injury is crucial. Proliferative adult α -cells have been captured by single-cell RNA sequencing and SHH signaling has been identified as a candidate pathway regulating α -cell proliferation [41,63,74].

Additionally, single-cell transcriptomics revealed species-specific differences in gene expression important to consider when designing new therapies for diabetes.

In conclusion, single-cell studies on pancreatic cells identified several genes and pathways critical for driving proliferation and maturation of β -cells and shed light on intra-endocrine cell heterogeneity, hint at underlying molecular mechanisms, and describe potential regenerative subpopulations and therapeutic targets.

6. FUTURE PERSPECTIVE

To understand the pathomechanisms of T1D and T2D and find potential routes to restore β -cell mass - the basis for diagnosis and therapy - we must determine the factors that shape β -cell identity, drive differentiation, and maintain function and plasticity in the pancreas. At the cellular level, these include the molecular phenotype and response mechanisms to external stimuli that regulate function, the spatial position and local environment, and the developmental history and maturation state [141]. Current studies almost exclusively have focused on descriptive analyses and statistical pattern identification, but they lack mechanistic insights. Single-cell analysis can infer function through correlation but will not substitute thorough functional validation by interventional analysis and perturbation experiments. New CRISPR-based technologies for gene manipulation in single-cells are promising approaches to obtain functional answers and fill the gap between the molecular profile and actual phenotype of a cell [142–145]. In addition, the transcriptome only hints at active processes and pathways but needs to be connected to a cell's proteome and epigenome to make conclusive statements on its

functional state and reveal post-translational mechanisms that can be targeted in therapy. Finally, and most importantly, the single-cell information needs to be mapped back onto healthy and diseased tissue as well as onto the temporal trajectory of differentiation. Imaging methods such as single-molecule RNA fluorescence *in situ* hybridization (FISH) [146,147] and imaging mass-cytometry [148] enable spatially resolved quantification of single mRNA molecules and proteins and connect a cell's phenotype to neighboring cells and the environment. Time-resolved data elucidate the genes controlling every single step in differentiation and maturation, important information lacking in end-point analyses. Single-cell methods and lineage-tracing in combination with organ on a chip approaches or *in vitro* differentiation approaches [7,149] will allow to follow the temporal progression of a cell, either live or in pseudotime reconstructed by machine learning algorithms.

Insights from pioneering single-cell studies of pancreatic cells have illustrated the potential of single-cell data. Now, we need to go beyond collecting data and simple descriptive, correlative analyses and integrate cellular profiles over multiple omics layers in space and time. This will pave the way to understand the mechanisms underlying diabetes and to design strategies for *in vivo* regeneration of functional islets.

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CONFLICT OF INTEREST

None declared.

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