Review

Interrogating islets in health and disease with single-cell technologies

Andrea C. Carrano [1,2], Francesca Mulas [1,2], Chun Zeng [2], Maike Sander [*,2]

ABSTRACT

Background: Blood glucose levels are tightly controlled by the coordinated actions of hormone-producing endocrine cells that reside in pancreatic islets. Islet cell malfunction underlies diabetes development and progression. Due to the cellular heterogeneity within islets, it has been challenging to uncover how specific islet cells contribute to glucose homeostasis and diabetes pathogenesis. Recent advances in single-cell technologies and computational methods have opened up new avenues to resolve islet heterogeneity and study islet cell states in health and disease.

Scope of review: In the past year, a multitude of studies have been published that used single-cell approaches to interrogate the transcriptome and proteome of the different islet cell types. Here, we summarize the conclusions of these studies, as well as discuss the technologies used and the challenges faced with computational analysis of single-cell data from islet studies.

Major conclusions: By analyzing single islet cells from rodents and humans at different ages and disease states, the studies reviewed here have provided new insight into endocrine cell function and facilitated a high resolution molecular characterization of poorly understood processes, including regeneration, maturation, and diabetes pathogenesis. Gene expression programs and pathways identified in these studies pave the way for the discovery of new targets and approaches to prevent, monitor, and treat diabetes.

© 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords Pancreatic islet; Endocrine cell; Single-cell; Heterogeneity; Type 2 diabetes; RNA-seq

1. INTRODUCTION

The islets of Langerhans in the pancreas contain five different endocrine cell types, namely beta, alpha, delta, gamma (also called PP cells), and epsilon cells, which each produce a different hormone. These hormones are secreted in response to metabolic cues and together orchestrate the maintenance of blood glucose homeostasis. Pancreatic hormones do not function in isolation, but influence each other's release through endocrine, paracrine, and autocrine feedback mechanisms [1,2]. It is well established that individual cells of a given islet cell type are heterogeneous in nature and that this heterogeneity forms an important basis for islet behavior [3]. Islet cell heterogeneity has been most extensively studied for beta cells, which play a critical role in the pathogenesis of diabetes. While it is well known that damage or loss of beta cells causes diabetes, how other endocrine cell types contribute to disease pathogenesis is not fully understood. Moreover, it is still largely unclear if different cellular states and subpopulations within islet cell types contribute to diabetes pathogenesis.

First observations that beta cells are heterogeneous and differ in regard to insulin secretion were made more than 30 years ago. Salomon and Meda developed methods to visualize insulin release from individual beta cells and reported substantial differences between individual cells [4]. The idea of functionally relevant heterogeneity among beta cells was further bolstered by studies showing that beta cell subpopulations exhibit different sensitivity to glucose [5,6] and change dynamically in response to glucose exposure [7—11]. It has been further suggested that the functional state of individual beta cells affects their fragility, as differences in insulin expression, glucose responsiveness, and oxidative state between beta cells have been associated with susceptibility to oxidative and cytokine-induced damage [12—14]. In the 1990s, the use of microscopy techniques and the development of fluorescent dyes greatly expanded the research of islet function with a strong visual impact at the single-cell level. Calcium imaging provided crucial information on the calcium influx pattern in response to glucose in different islet cell types [15—17]. Measurement of cytosolic calcium in individual beta cells further revealed heterogeneity of calcium oscillations in response to different secretagogues among individual beta cells [18]. One shortcoming of these early studies was that they were conducted in dispersed islet cells and therefore lacked spatial resolution and presence of functionally relevant cues in the intact islet. Recent studies, using optical interrogation of intact islets in tissue slices have overcome these limitations and have convincingly demonstrated functional differences

Departments of Pediatrics and Cellular & Molecular Medicine, Pediatric Diabetes Research Center, University of California, San Diego, 2880 Torrey Pines Scenic Drive, La Jolla, CA 92037, USA

[1] Andrea C. Carrano and Francesca Mulas contributed equally to this work.
[2] Fax: +1 858 246 0162.

*Corresponding author. E-mail: masander@ucsd.edu (M. Sander).

Received February 18, 2017 • Revision received April 10, 2017 • Accepted April 11, 2017 • Available online 5 May 2017

http://dx.doi.org/10.1016/j.molmet.2017.04.012

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

between individual beta cells in rodent and human islets [19–21]. In addition to insulin secretion, heterogeneity among beta cells also exists for other features. For example, beta cells differ with regard to their proliferative activity [22,23] and expression of senescence markers, such as p16Ink4a [24,25].

Until recently, most of our knowledge about how islet cells change their molecular features in response to different physiological and pathological conditions was obtained by studying whole islets. As these studies detect global patterns, they represent an average dominated by the most abundant cell types and thus mask contributions from more rare cell types and subpopulations. Fluorescence-activated cell sorting (FACS)-enrichment has been utilized to study alpha, beta, and delta cell populations. In mice, this has been accomplished by genetically tagging endocrine cell populations and isolating these cells based on a fluorescent reporter [26–29]. This strategy, however, cannot be applied to human cells, and sorting strategies for the less abundant gamma and epsilon cells are not available. In humans, other methods, such as cell sorting based on surface markers or intracellular proteins, have been used to obtain cell type-specific transcriptomes of islet cells [30–36]. But even with sorted cells, population-based profiling of islet cell types masks the variation across individual cells, thus limiting insight into different cell states or subpopulations. Recently, cell sorting strategies have been developed that can separate beta cell subpopulations in both mice and humans based on expression of specific marker genes [23,31]. Molecular analysis of these subpopulations has revealed differences with regard to proliferative capacity and responsiveness to nutrient cues. Grompe and colleagues identified distinct beta cell populations in humans that exhibit differences in basal and stimulated insulin secretion, as well as gene expression profiles [31]. The relative abundance of these subpopulations was found to be significantly altered in islets from donors with type 2 diabetes (T2D). It may be that the different subtypes influence pathogenesis through differences in susceptibility to metabolic stress, proliferative capacity, or maturation state.

Over the past decades, we have amassed a wealth of knowledge about islet function in health and disease, employing electrophysiological, microscopy, genetic, and population-based gene expression profiling approaches. More recently, an abundance of different single-cell technologies has been developed that allows even higher-dimensional analyses of isolated single cells [37–39]. These new techniques have been applied to islet cells, as demonstrated by multiple papers published on this topic in the last year, and represent a breakthrough in islet biology and diabetes research. In this review, we summarize what we have learned from studying islets at the single-cell level using new single-cell technologies to investigate islet cell function, physiology, and pathogenesis (Figure 1). Moreover, we highlight current challenges encountered when analyzing the high-dimensional data obtained using new single-cell technologies, as well as discuss how these new technologies can be utilized in the future to further interrogate islets.

2. NEW SINGLE-CELL METHODOLOGIES TO STUDY ISLET CELL PHYSIOLOGY AND FUNCTION

Just in the past year, multiple studies using new single-cell technologies have been published with the goal to understand how specific pancreatic cell types contribute to glucose homeostasis and diabetes pathogenesis (Table 1). These studies analyzed different tissues, including whole pancreas and isolated islets from mouse and human in healthy and diabetic conditions. The studies varied greatly in regard to the experimental technology used to generate single-cell expression data as well as applied methods for data analysis. To capture individual cells from pancreatic tissue, flow cytometry [40–44] and microfluidic methods [45–49] were utilized. While the most common technology used to study the molecular profile of individual pancreatic cells has been RNA-seq [41–49], proteomic approaches including imaging mass spectrometry (IMS) and mass cytometry have also been applied [40,50]. Recent advancements in computational methods and the development of new algorithms to reconstruct and investigate molecular processes has aided in reducing these high-dimensional data to an interpretable form. By revealing the cellular heterogeneity found in the pancreas, studies using single-cell technologies have advanced our understanding of cell function and cell communication in the pancreas. The technologies enabled the study of less abundant islet cell types and revealed previously unknown cellular states and subpopulations of endocrine cells. Together, these studies have uncovered new functions for islet cells and allowed a high-resolution molecular characterization of poorly understood processes, including islet cell regeneration, maturation, and T2D pathogenesis.

2.1. Adult islet cell function

Single-cell profiling allows the interrogation of less abundant endocrine cell types, which have remained elusive in studies of both whole islets and sorted islet cells. Multiple studies from this past year that have uncovered the transcriptional signatures of individual human delta, gamma, and epsilon cells from human islets suggest important and novel roles for each islet cell type in sensing and integrating specific systemic cues to govern islet function [42,43,47]. These novel insights are based on the observations that receptors for cell signaling pathways are specifically enriched in individual islet cell types (Figure 1). Compared to other islet cell types, delta cells, for example, highly express receptors for leptin (*LEPR*) and ghrelin (*GHSR*) [42,43,47], suggesting that pancreatic responses to these appetite-regulating hormones are mediated by these cells. Indeed, work by Huising and colleagues has recently demonstrated that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets [27]. Delta cells also exhibit high expression of receptors for specific neurotransmitters (dopamine; *DRD2*) and growth factors (*ERBB4*) [43,47], while gamma cells selectively express receptors for acetylcholine and serotonin [42,47]. Epsilon cells, which make up less than 1% of endocrine cells, also uniquely express various receptors for neurotransmitters, endorphins, prostaglandins, and glycoproteins [43]. Thus, single-cell analysis has uncovered novel roles for these rare islet cell types as integrators of systemic cues and metabolic signals in the islet.

While much has been uncovered studying transcriptomes of the less abundant endocrine cell types, novel insight gained by single-cell RNA-seq analysis into the function of beta and alpha cells has been more limited. Genes displaying high expression in beta or alpha cells overlapped largely with those found in previous transcriptome studies employing cell sorting methods to isolate beta or alpha cells from mouse and human islets [43,47–49]. While the observation that beta cells express genes associated with glucose sensing, uptake, and metabolism confirmed prior studies, the single-cell analysis revealed a previously not fully appreciated heterogeneity in gene expression among individual beta cells. Previous studies provided evidence for beta cell heterogeneity both at a functional and gene expression level [21,23,31,51]; however, the extent of this heterogeneity could only be fully resolved by single-cell analysis. Using computational methods, such as principal component analysis (PCA) and t-Distributed Stochastic Neighbor Embedding (tSNE) to visualize groups of cells with similar transcriptional profiles, recent single-cell studies have

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

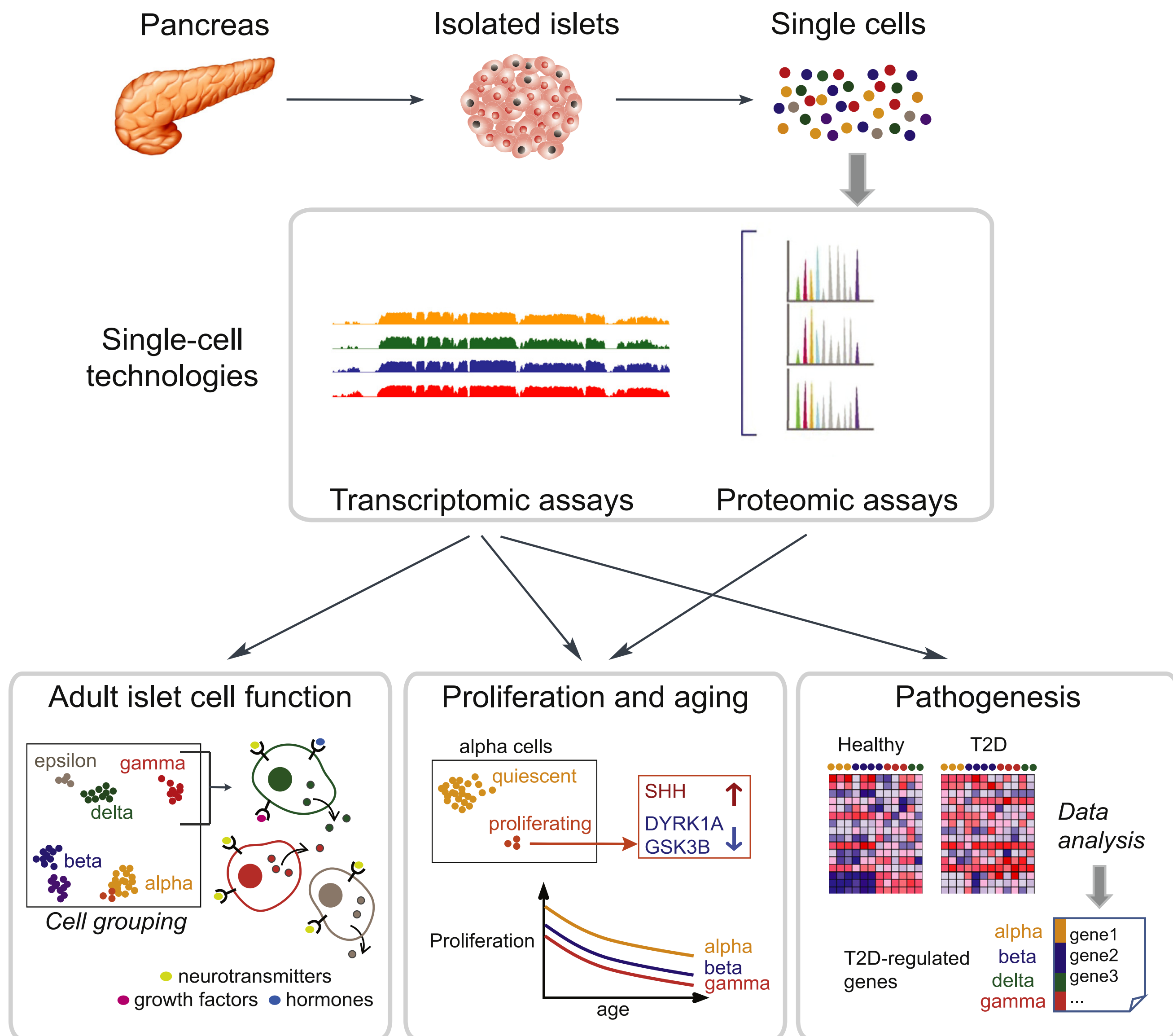

Figure 1: Single-cell approaches to interrogate the transcriptome and proteome of islet cell types in health and disease. Transcriptomic and proteomic studies of single islet cells have provided novel insights into islet cell function, proliferation and aging, and type 2 diabetes (T2D) pathogenesis. For example, single-cell studies have identified receptors for neurotransmitters, growth factors, and hormones specifically expressed in epsilon, delta, and gamma cells (bottom, left panel), suggesting these rare islet cell types integrate systemic cues and metabolic signals. Identification of transcriptionally distinct subpopulations of beta cells (bottom, left panel) and alpha cells (bottom, left and middle panels) has provided insight into different functional states of endocrine cells as well as enabled profiling of rare proliferating cells. Proteomic profiling of single islet cells has shown that multiple endocrine cell types exhibit reduced proliferation with age (bottom, middle panel). Finally, single-cell profiling has uncovered differentially expressed genes in islet cells from healthy and diabetic individuals (bottom, right panel), showing contribution of multiple endocrine cell types to islet pathophysiology and revealing novel genes and pathways with potential for therapeutic targeting.

identified multiple subpopulations of beta cells in healthy adult human donors (Figure 1). For example, Sandberg and colleagues identified five distinct subpopulations of human beta cells by comparing expression values of the fifty most variable genes in these cells. While each population had similar insulin levels, they differed in levels of specific transcriptional regulators, fatty acid receptors, and adipokines [43]. For example, two of the subpopulations identified expressed *RBP4*, an adipokine found elevated in insulin-resistant mice and humans with obesity and/or T2D [52]. These same populations also expressed the free fatty acid receptor *FFAR4 (GPR120)*, which has been implicated as a regulator of apoptosis in human islets [53]. What distinguished these two populations was expression of the helix-loop-helix transcriptional repressors *ID1*, *ID2*, and *ID3*, loss of which has been shown to enhance insulin sensitivity and protect mice against diabetes [54—57]. Likely, the different populations found in this study have different properties regarding their response to metabolic stress and susceptibility to diabetes. Interestingly, depending on the experimental setup and the analytical methods used, the number of subpopulations and the genes that identified each population varied between studies. For example, other studies with human islets identified subpopulations based on differences in expression of oxidative and endoplasmic reticulum (ER) stress response genes [42,45] Notably, some groups did not find evidence for beta cell subpopulations [47,49]. Interestingly, while subpopulations were found among beta cells, no subpopulations were

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1 — Summary of recent studies using single-cell approaches to study pancreatic islets.

Single-cell method	Cell source	Key findings in islets	Reference
RNA-seq	Pancreatic cells from 4 human adult donors	- Identified cell-type-specific genes for alpha and beta cells linked to T2D - Observed subpopulations of beta cells distinguished by genes implicated in ER and oxidative stress response - Found delta and gamma cells express multiple receptors for cell signaling pathways	Muraro et al., 2016 (Ref. [42])
RNA-seq	Pancreatic cells from 10 healthy and T2D adults	- Observed subpopulations of alpha and beta cells - Found delta and epsilon cells express multiple receptors for cell signaling pathways - Identified genes differentially regulated between healthy and T2D donors in alpha, beta, and gamma cells	Segerstople et al., 2016 (Ref. [43])
RNA-seq	Human islets from 8 healthy and T2D adults	- Found delta and gamma cells express multiple receptors for cell signaling pathways - Identified genes differentially regulated between healthy and T2D donors in alpha, beta, and delta cells	Lawlor et al., 2017 (Ref. [47])
RNA-seq	Human islets from 18 healthy and T2D adults	- Identified 245 genes dysregulated in endocrine cells from T2D donors - Observed high degree of similarity between the islet cell types	Xin et al., 2016 (Ref. [48])
RNA-seq	Mouse islets	- Observed high degree of similarity between the islet cell types	Xin et al., 2016 (Ref. [49])
RNA-seq	Mouse beta cells over postnatal time course	- Obtained high-resolution map of beta cell transcriptome dynamics after birth - Demonstrated role for amino acids and ROS in postnatal beta cell proliferation	Zeng et al., 2017 (Ref. [44])
Mass cytometry	Human islets from 20 donors (including children and healthy and T2D adults)	- Confirmed exponential decline in beta cell proliferation after childhood - Found alpha cells have the highest basal replication of all endocrine cell types - Identified three different cellular states of beta cells - Found islet cell composition is partially age-dependent	Wang et al., 2016 (Ref. [40])
RNA-seq	Human islets from 9 donors (including children and healthy, T1D, and T2D adults)	- Identified role for sonic hedgehog signaling in alpha cell proliferation - Found alpha and beta cells from T2D donors have expression profiles with features similar to juvenile beta cells	Wang et al., 2016 (Ref. [61])
RNA-seq	Beta cells from 3- and 26- month old mice	- Found beta cells from old mice have transcriptional profiles similar to those of young mice	Xin et al., 2016 (Ref. [46])
RNA-seq	Pancreas cells from 4 human adult donors and 2 mouse strains	- Detected subpopulations of beta cells characterized by levels of ER stress	Baron et al., 2016 (Ref. [45])
RNA-seq	Human islets from 1 adult donor	- Observed beta cell- and endocrine-specific expression of genes associated with diabetes risk found in GWAS	Li et al., 2016 (Ref. [41])
MALDI MS	Rat islets	- Observed heterogeneity in cell composition between islets based on location in pancreas	Jansson et al., 2016 (Ref. [50])

uncovered within delta, gamma, and epsilon cells; however, a small population of human alpha cells distinguished by high expression of proliferation-associated genes was found in one study [43]. The discovery of these different subpopulations within islet cell types may offer important clues for understanding the functional status of endocrine cells. Experiments designed to follow these subpopulations over time could provide important insight into the flexibility of individual islet cells to cycle between different functional states.

2.2. Islet cell proliferation and plasticity

It is well established that beta cell proliferation capacity declines rapidly and severely with age [58,59]. Indeed, recent studies applying single-cell RNA-seq in mice of different ages confirmed that as mice age, there is a reduction in expression of cell cycle pathway genes in beta cells [44,46]. However, it is unknown if other endocrine cells also lose their capacity to replicate during adult life. To investigate this, Kaestner and colleagues used single-cell mass cytometry to identify proliferating cells in islets from twenty human donors aged 18 days to 65 years [40]. Mass cytometry, a technology that uses heavy metal ions as labels for probes such as antibodies, rather than using fluorochromes, enables the investigation of cell identity and behavior at the level of proteins [60]. By labeling human islet cells with metal-conjugated antibodies against the proliferation marker Ki67 along with specific cell markers and measuring antibody abundance within each cell using time-of-flight spectrometry, the group showed that similar to beta cells, alpha and delta cells also exhibit reduced proliferation with age (Figure 1).

Because endocrine cells replicate at a very slow rate, it has been exceedingly difficult to study proliferating endocrine cells at the molecular level. Single-cell technologies have enabled transcriptional profiling of these rare cells. Recent studies have begun to elucidate differences of proliferating and non-proliferating alpha cells [43,61]. These studies have shown that proliferating alpha cells are transcriptionally distinct from non-proliferating alpha cells (Figure 1). For example, from healthy donors and donors with T2D, Sandberg and colleagues identified twelve alpha cells that separated from other alpha cells with two-dimensional data reduction by t-SNE [43]. These constituted proliferating cells that were distinguished by a signature of 439 significantly upregulated genes consisting mainly of cell cycle regulators. Additionally, based on high transcript expression of the proliferation marker Ki-67, Kaestner and colleagues identified one proliferating alpha cell in their single-cell RNA-seq profiling of healthy

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

human adult pancreas samples [61]. By comparing the profile of this lone proliferating cell with quiescent alpha cells from the same donor, they found that the sonic hedgehog (SHH) mitogenic pathway was only activated in the proliferating cell. Moreover, both *DYRK1A* and *GSK3B,* regulators of GLI transcription factors downstream of SHH signaling, were repressed in the proliferating cell. This is consistent with studies showing that inhibition of DYRK1A and GSK3B, using harmine and aminopyrazine compounds, induces beta cell proliferation [62,63]. Profiling of proliferating islet cells provides clues to understanding mechanisms of islet cell regeneration; however, the extent of similarity between pathways activating alpha and beta cell proliferation remains to be determined.

While transcriptomes of single proliferating human alpha cells have been obtained from these studies, proliferating beta cells in the adult human pancreas have yet to be captured. Likely, the total number of single cells procured and analyzed in these studies has been too low to obtain signatures of these very rare cells. The single cell mass cytometry study by Kaestner and colleagues indicates that when compared to the other endocrine cell types, alpha cells have the highest basal replication rate in the adult islet (Figure 1) [40]. This high rate of replication in alpha cells could be exploited to utilize alpha cells as a source for new beta cells via cell fate conversion [64]. Indeed, single-cell RNA-seq studies in both mouse and human islets have shown that transcriptional profiles of alpha and beta cells exhibit striking similarity, with only 26 genes being specifically enriched in alpha cells and 151 genes in beta cells [48,49]. The high degree of similarity between these two cell types suggests that a small number of genes control cell identity, which may explain why under conditions of extreme beta cell loss, alpha cells spontaneously transdifferentiate into beta cells [65]. Now, recent studies show that stimulation of $GABA_A$ receptor signaling can induce alpha-to-beta cell conversion in rodent and possibly also human islets [66,67], suggesting the existence of druggable targets for the regeneration of beta cell mass from alpha cells.

2.3. Beta cell replication, functional maturation, and aging

Recent studies have shown an association of beta cell replication with reduced expression of genes that define the main function of adult beta cells, namely the production and release of insulin in response to glucose [68]. Beta cell replication capacity rapidly declines with age, and this process has been shown to coincide with increased beta cell secretory function [25,28,69]. To begin to understand how transcriptional differences contribute to improved secretory function with age, a few groups have compared transcriptomes of individual beta cells from young and aged rodents and humans. In one study, Gromada and colleagues performed single-cell RNA-seq on beta cells from 3- and 26-month-old mice and found that beta cells from very old mice have a similar gene expression signature as beta cells from young mice, with the exception of a small number of genes encoding transcription factors, cell cycle regulators, and regulators of cell death [46]. However, by three months of age, proliferation rates are already quite low in mice [59] and almost comparable to aged mice. This may explain why studies comparing transcriptomes of sorted beta cell populations from mice at 4—6 weeks of age and 16—20 months of age uncovered a greater number of differentially expressed genes [28]. Another single-cell transcriptome study of human pancreas cells compared endocrine cells from children as young as 19 months to adults and found that beta and alpha cells in children are more similar to each other than adult alpha and beta cells [61]. Specifically, many alpha cell signature genes identified in adult alpha cells were found to be expressed in juvenile beta cells. Likewise, beta cell signature genes were expressed in juvenile alpha cells. As gene set enrichment scores for adult endocrine cell signatures were lower in juvenile alpha and beta cells, this would indicate these cells were in the process of maturing to a fully functional state.

After birth, pancreatic endocrine cells achieve a fully differentiated state after completion of a maturation process, which takes place in the early postnatal period [70]. The steps toward beta cell maturation, which likely involve changes in gene expression programs, had been poorly understood. To probe this process, our group generated single-cell RNA-seq data of mouse beta cells from multiple early postnatal time points [44]. Then, to obtain a high-resolution map of beta cell transcriptome dynamics after birth, we ordered beta cells based on transcriptional similarity using 1D-PCA. The method allowed the exploration of gene patterns over a reconstructed early postnatal developmental trajectory and exposed previously unrecognized transcriptional dynamics of maturation. For instance, we found that immature, proliferative beta cells exhibit high expression of regulators of amino acid metabolism and mitochondrial activity as well as a network of nutrient responsive transcription factors [44]. Reduced expression of these components correlated with diminished proliferation with age. Furthermore, experimentally reducing levels of mitochondrial reactive oxygen species during postnatal beta cell development led to lower beta cell proliferation rates and reduced beta cell mass. The high expression of components of the mitochondrial respiratory chain in proliferating beta cells is consistent with an earlier study by Dor and colleagues comparing transcriptomes of sorted proliferating and quiescent beta cells [68]. Moreover, mild ER stress has been shown to promote beta cell proliferation [71] and the transcription factors found in our study are downstream effectors of this pathway. Interestingly, using a method based on 1D-PCA, Yanai and colleagues found subpopulations of beta cells in healthy human donors that were distinguished by high expression of either ER stress-inducible genes (i.e. *HERPUD1, HSPA5,* and *DDIT3*) or functional genes (*CHGA, UCN3, NEUROD1,* and *MAFA*) [45]. Subpopulations with high expression of ER stress genes and low expression of functional genes could represent beta cells that are proliferating or that have the potential to proliferate. Supporting this idea, our study of single mouse beta cells during early postnatal development identified ER stress markers as enriched in proliferative beta cells [44]. The combined evidence from these studies suggests that improved function of beta cells, as seen in the course of beta cell maturation and aging [25,72,73], is at the expense of regenerative potential. By revealing novel pathways regulating beta cell maturation and proliferation, these studies have important implications in identifying therapeutic targets to stimulate beta cell regeneration.

3. SINGLE-CELL PROFILING TO UNDERSTAND MECHANISMS OF ISLET PATHOPHYSIOLOGY AND DIABETES

Identifying gene expression programs and pathways that contribute to islet dysfunction and diabetes are a critical step toward identifying drug targets and approaches to prevent, monitor, and treat diabetes. Several studies have reported differences in gene expression profiles between islets from normoglycemic individuals and those with T2D [32,53,74,75]. However, the observed differences are difficult to interpret, because these studies performed transcriptome analysis of whole islets. The population-based analysis leaves unclear how individual cell types or subpopulation of cells within a particular islet cell lineage contribute to the observed differences.

One challenge that has made it difficult to compare gene expression profiles of whole islet preparations from normal and diabetic

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

individuals is the substantial variation in islet cell type composition among human islets from different donors [76]. An additional obstacle is that the proportion between islet cell types is altered in T2D [77–79]. The observation that human islets are heterogeneous with regard to cell type composition has recently been confirmed by single-cell RNA-seq studies [43,47]. Moreover, using single-cell mass cytometry, Kaestner and colleagues found that donor-to-donor variability in cellular composition is partially age-dependent [40]. To enable a meaningful comparison of whole islet RNA-seq data from healthy and T2D individuals, Yanai and colleagues developed a computational method to resolve cell type heterogeneity by deconvolving bulk RNA-seq data sets [45]. To estimate the proportion of each cell type in the whole islet preparations, their method utilizes single-cell gene expression data to identify primary defining genes specific for each cell type. Importantly, applying this method, they found that a large number of genes identified in bulk transcriptome analysis as differentially expressed between diabetic and healthy donors is likely variable due to cell type composition differences between donors, leading to false assumptions about differential gene expression in beta cells from healthy and diabetic individuals. Similarly, differential expression of some genes between these two groups was masked by the cell population differences. Using the algorithm to correct for cell population differences, the authors noted cell type-specific effects of the hyperglycemic state on gene expression in alpha and beta cells. The analysis revealed upregulation of functional genes, such as *UCN3* and *NEUROD1,* in alpha cells and downregulation of ER stress-related genes in beta cells.

Single-cell RNA-seq studies also suggest that, in addition to beta cells, other endocrine cell types may contribute to islet pathophysiology [41,43,47,48]. For example, genes linked to rare and common forms of islet dysfunction and diabetes were found to be expressed in human delta and gamma cells [47]. Additionally, several genes associated with increased risk for the development of diabetes, identified through genome-wide association studies (GWAS), were found to have differential expression between human endocrine cell types [41,47]. A potential contribution of other endocrine cell types, including alpha, delta, and gamma cells, to diabetes pathogenesis was also suggested by differential expression studies comparing transcriptomes of single islet cells from healthy and T2D donors [43,47]. Gene set enrichment analysis (GSEA) showed that genes controlling energy metabolism in mitochondria and protein synthesis were significantly downregulated in most cell types in individuals with T2D, while genes linked to apoptosis, diabetic nephropathy, and cytokine signaling were upregulated [43]. Novel genes not previously implicated in diabetes or islet function, including genes encoding GPCRs, ion channels, transcription factors, as well as lncRNAs, were also identified in these studies. The biological significance of these gene regulatory differences in T2D pathogenesis remains unclear and further investigation of the roles of these genes in non-beta islet cell types is needed.

Looking at differentially expressed genes in healthy and diabetic beta cells may also offer clues on causes of beta cell death in the diabetic state (Figure 1). RNA-seq studies from Sandberg and colleagues have shown that in beta cells the most significantly downregulated gene is the ion transporter regulator *FXYD2* [43]. Loss of this beta cell-specific gene has been previously associated with loss of insulin-expressing cells in patients with type 1 diabetes (T1D) [80], suggesting common mechanisms regulating beta cell death in these two types of diabetes. This same study also found that beta cells from donors with T2D showed upregulation of genes associated with metabolism, such as *GPD2* and *LEPROTL1*/*Endospanin-2*. GDP2, which catalyzes the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate using FAD as a cofactor, couples glycolysis with activation of mitochondrial energy metabolism to trigger insulin secretion [81]. LEPROTL1/Endospanin-2 has been shown to regulate cell surface expression of the leptin receptor, which is key for modulating energy balance and body weight [82]. It may be that dysregulation of these genes changes metabolic activity of beta cells and affects how beta cells respond to environmental cues, contributing to their dysfunction. Previous studies have suggested that beta cell dedifferentiation may underlie beta cell loss in T2D [83–85]. Indeed, Kaestner and colleagues' single-cell study suggests that this might be the case [61]. They found that beta cell gene signatures from diabetic adults had expression profiles similar to those seen in children. The immature gene expression signature of beta cells in the diabetic state included misexpression of cell cycle and insulin secretory genes, suggesting that beta cells from individuals with T2D are not able to maintain a fully differentiated gene expression profile. Notably, a study by Stitzel and colleagues came to a different conclusion; they found no transcriptional evidence for dedifferentiated beta cells in T2D islets [47]. In their analysis of single-cell transcriptomes from T2D and healthy islet cells, there was no significant difference in the expression of genes reported to be differentially regulated during dedifferentiation, including *FOXO1, NANOG*, and *POU5F1* [83]. Moreover, they did not observe significant shifts in islet cell populations or increases in numbers of hormone-negative cells. It may be possible that dedifferentiated cells were not captured in the latter study and that further studies are needed to determine if beta cell dedifferentiation does indeed occur in T2D.

The use of new single-cell technologies to study transcriptional alterations in T2D not only has pointed to novel molecular contributions of specific islet cell types in diabetes but also has provided clues about possible pathways and mechanisms associated with diabetes pathology. Further studies validating the genes and pathways revealed by single-cell studies as candidates for therapeutic targeting are likely on the horizon.

4. CHALLENGES OF SINGLE-CELL ANALYSIS

Single-cell technologies have the potential to greatly improve our understanding of how individual cells contribute to islet function and pathogenesis by providing unprecedented access to unique transcriptomic and proteomic signatures. However, there are many challenges in analyzing the resulting high-dimensional, complex data sets. For both transcriptomic- and proteomic-based analysis of single-cell data, there are no established standards for data pre-processing and noise removal, which can affect interpretation of the data in significant ways. Methods to cope with the inherent noise and the high dimensionality of single-cell data are still being evaluated, and proper application of these strategies is crucial to not only discern genuine gene expression from technical artifacts but also to compare data across different laboratories and instruments. While multiple papers have reported methods to analyze transcriptomic and proteomic single-cell data [86–88], the best use of these novel bioinformatic tools is still a subject of debate.

One major challenge facing single-cell RNA-seq data analysis is overcoming unwanted sources of variation that originate from amplification biases in the sequencing protocol and/or experimental variability. This is key to correctly interpreting results, and, despite the numerous approaches developed to remove these unwanted factors, it remains a non-trivial problem. To estimate technical sources of variation arising during amplification of low input material amounts, several islet single-cell studies have taken advantage of External RNA Control Consortium (ERCC) spike-in controls, a set of 92 RNAs with

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

different lengths and concentrations that are carried along through the library preparation and sequencing process of individual cells [41,42,61,89]. However, exogenous spike-in standards have several caveats, including a shorter RNA length compared to average human mRNAs, which may result in transcription efficiency bias [89]. In fact, the ratio of reads mapped to the spike-ins is often indicative of biologically relevant variability instead of quality, especially when dealing with cells at different stages of the cell cycle [89]. To overcome these problems, other approaches have been proposed to estimate technical noise and normalize data in the absence of spike-ins [86,90]. These methods compute the coefficient of variation (CV) for each gene to define noisy genes. By definition, the CV is inversely proportional to the mean expression value of a gene. Genes with high CV values reflect heterogeneity within the cell population under study, while CV values below a certain threshold would be considered noise. However, this can be problematic when analyzing gene expression data from endocrine cells, as *INS*, *PPY*, and *SST* transcripts alone account for more than 50% of the total cellular transcripts in beta, gamma, and delta cells, respectively [43], and the high expression values result in low CV values for these genes. Thus, a major challenge in the application of these noise reduction approaches to the study of endocrine cells is the identification of a suitable threshold of CV that would enable noise removal while retaining key cellular identity genes in the analysis.

Reliable and more conservative approaches based on surrogate variable analysis (SVA) have been employed in many contexts and can be used to identify batch effects and other hidden sources of unwanted variation [87,88]. SVA distinguishes sources of expression variation that are due to technical artifacts (surrogate variables) from the biological signal. Given a model specifying the variables of interest (i.e. disease states), SVA identifies genomic data affected by artifacts and uses a mathematical model to adjust the data for subsequent analysis. As the estimated variability may include differences due to cell cycle or other biological factors, a careful analysis of both the parameters used and expected cell groupings or gene patterns is crucial to define a model for noise estimation that best fits the study. Indeed, cell cycle or other variables can be treated as confounding factors and can be explicitly modeled prior to gene pattern analysis [91]. Recently, a similar approach was used by Sandberg and colleagues to model the "donor effect", representing the variability of pancreatic samples coming from different human donors [43]. As discussed above, the authors identified a group of proliferating alpha cells distinguished by a gene signature of cell cycle regulators. Notably, this group of cells was not detectable before removing the donor effect with Combat, a batch effect removal tool that is particularly powerful in analyzing low sample size data [92]. Methods for SVA can also be used to normalize samples collected under different conditions, a step particularly important when comparing single-cell data with publically available datasets. Using Combat, Kubicek and colleagues were able to directly compare the different pancreatic cell types under study with previously published data in a single multidimensional scaling plot [41]. While comparison with published data is extremely useful to assess the validity of an experiment, it emphasizes the need for data rescaling and batch effect removal approaches to eliminate the effects of different experimental settings underlying each separate dataset.

Another major challenge in single-cell studies is the visualization and extraction of biologically relevant patterns in high-dimensional data. In order to visualize cell-to-cell similarities among islet cell types and to distinguish subpopulations with a similar transcriptional signature, most studies took advantage of dimensionality reduction techniques such as PCA, tSNE, and other clustering methods [41—43,45,48,49]. Using these methods, cells can be assigned into groups (cell-type calling) based on their expression of cell-specific marker genes. One significant challenge has been to define cells whose transcriptome or proteome do not cluster with other groups, e.g. those forming isolated groups in PCA plots or placed on distinct branches of a hierarchical clustering dendrogram. The function of these cells could be investigated by examining genes that best distinguish them, as defined by differential analysis or highest loadings genes with PCA analysis. However, given the limited number of cells for analysis, conclusions should be drawn with a degree of caution. In addition, the use of different clustering approaches to perform cell type calling as well as lack of established tools for noise reduction may pose an issue in reproducibility across studies. This has been demonstrated in recent studies that have identified subpopulations within the islet cell types. While some studies showed the existence of alpha and beta cell subpopulations with distinct gene signatures [42,43,45], others did not observe existence of such subpopulations [47,49]. The increasing number of studies with large sample sizes and the availability of accurate comparative methodologies should help to assess which subpopulations have a consistent role in islet biology. For instance, more reliable clusters of endocrine cells could be obtained using methods robust to missing data (such as model-based clustering) or suitable for binary data (based on Jaccard distance) [37]. One cannot be certain that the distinct subpopulations found are an artifact of experimental or clustering methods. Additional validation of the groupings could be obtained by systematically comparing multiple datasets using the same analytical pipeline after proper batch effect removal [41]. In addition, computational methods to assess the optimal number of clusters, such as the Silhouette index, could be exploited to further evaluate different cell groupings.

Dimensionality reduction techniques can also be used to infer trajectories of cellular events, as demonstrated by ordering cells according to their maturation level, as shown in beta cells [44] and in other contexts [90,93,94]. In the past two years, methods to infer a temporal trajectory of single cells by analyzing transcriptomes have become available [94]. While these tools could be useful for studying islet cells, the lack of intuitive tools for some of the methodologies and the limited guides that help biologists identify the most suitable approach for their data make the use of ordering methods challenging. To facilitate the comparison of cell orderings obtained with different methodologies, Ji and Ji have developed the "Pseudotemporal Ordering Score" (POS) [94]. However, the POS cannot be applied to all methods; therefore, additional methods for comparison of cell ordering results are needed.

The high-dimensional nature of data obtained from single-cell proteomic datasets also possess challenges in analyzing data bioinformatically. Single-cell mass cytometry studies have enabled the study of proliferation rates of pancreatic endocrine cells [40]; however, this method could help address many other questions. For instance, mass cytometry data have been explored to identify novel cell subtypes [95], to examine progression of cellular programs [96], or to analyze cellular networks [97]. To interpret the complex dynamics underlying mass cytometry data, bioinformatic approaches using visualization strategies are required. Among those discussed, tSNE is an intriguing method that is becoming widely used for the analysis of all types of cytometric data [98]. As implemented in the software viSNE, this method maps cell-to-cell similarity in a two-dimensional space without assigning cells to mutually exclusive clusters. However, wide spread use of these advanced methods may be limited because they require significant computational resources.

Taken together, the outlined challenges demonstrate that in order to obtain biologically relevant results, researchers with biological and

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

computational backgrounds need to collaborate when analyzing single-cell data. In the future, new methodologies and computational strategies are required to fully exploit high-resolution data from single cells. The development of such methods will facilitate further exploration of data from individual islet cells for a better understanding of gene and protein regulation in islet health and disease.

5. PERPECTIVES AND FUTURE OUTLOOK

While the recent surge of single-cell studies on pancreatic cells has improved our understanding of pancreatic cell function, the studies have shed little light on how transcriptomic and proteomic landscapes are spatially organized throughout the pancreas. Single-cell studies of pancreatic cells so far have used dissociation approaches to capture single cells, removing information about a cell's original spatial context and cellular environment. New imaging-based technologies now allow us to gain spatial resolution while measuring the transcriptome or proteome of single cells. Imaging-based techniques, including smFISH [99,100], Padlock probes and RCA [101,102], and Branched FISH [103,104], are technically easier than other high-throughput techniques and enable robust quantification of a small number of transcripts in intact tissues through fluorescently labeled probes. With recent advances using sequential barcoding or multiple probes with smFISH and Branched FISH, it is now possible to identify many transcripts in a single cell [105]. In order to quantify a large number of transcripts, more complex sequencing-based approaches have been proposed that enable RNA extraction from discrete regions or anatomical locations. One of these technologies, TIVA (transcriptome in vivo analysis), was recently used to profile the transcriptomes of single neurons from intact brain tissue. From this study, the authors were able to demonstrate that the tissue microenvironment shapes the transcriptomic landscape of individual cells [106]. Such characterization is also possible at the protein level using approaches based on mass cytometry. Landmark methods combining high-resolution laser ablation with mass cytometry to simultaneously measure cell distance and protein levels by mass spectrometry have succeeded in coupling protein expression with information on cell location in the tissue [107]. Crucial spatiotemporal interdependencies could also be revealed using these spatially resolving technologies when evaluating multiple time points though maturation or disease progression. For example, spatial information can be combined with reconstructed temporal profiles of cells, similar to the pseudotime scale generated in our study of beta cell maturation dynamics [44]. If the spatial location of a cell is known, patterns of gene or protein expression can be discerned, which will help identify relevant cell-cell signaling circuits in complex tissues. To this end, methods have been developed to cluster cells using both spatial and quantitative information via a Markov random field (MRF)-based approach [108]. MRF is a statistical method that enables modeling entities composed of multiple discrete sites, such as a biological tissue, with sites corresponding to cells. An MRF model can be represented as a graph in which each cell is a node that is linked to the immediate neighbors in the tissue. From this, an optimal clustering structure that preserves both gene expression similarity as well as spatial dependencies between cells can be obtained. As demonstrated, methods that directly include spatial coordinates in the clustering scheme are more likely to identify biologically relevant cell signatures compared to approaches that only consider transcriptomic information [108].

Single-cell RNA-seq has enabled the identification of cell types and subpopulations based on characteristic gene expression patterns; however, populations can also be distinguished by distinct patterns of accessibility and modification of regulatory DNA sequences [109–111]. Recent advances in single-cell ChIP-seq technologies now allow mapping of histone modifications at single-cell levels with reduced background noise to uncover genomic features and transcriptional states of individual cells [112]. Other novel deep sequencing assays, such as single-cell ATAC-seq and single-cell Hi-C, can explore variation in chromatin accessibility between single cells [113–115]. Single-cell ATAC-seq interrogates the entire genome for accessibility to DNA binding proteins, providing insights into cell-to-cell variation with regard to specific trans-factors and cis-elements that determine three-dimensional genome organization [114]. Moreover, single-cell Hi-C explores interdomain contacts within chromosomes to capture the conformation of individual cell genomes [113,115]. These methods shift average estimates of chromosome architecture from a population of cells to an exact quantification for each cell, ultimately providing information on variability in genome activity patterns.

Integrative approaches that combine different data sources are needed to obtain a complete picture of the differences between cell types and subpopulations within the pancreas. If used in combination, these methodologies would allow the exploration of how molecular features, including chromatin, gene expression, and/or protein expression, are co-regulated in both time and space. In addition to approaches integrating spatial coordinates with transcriptomic data discussed above, efforts have been made in other fields to combine genome and transcriptome information from the same cell [95,96,116,117]. Similarly, integrated single-cell approaches can be applied to study pancreatic cells. For example, proteomic data from single-cell mass cytometry studies showing inter-islet differences based on location in the pancreas [50] can be combined with transcriptomic and genomic data to determine how individual islets differ and if these features are of functional significance. With the popularity and potential of single-cell research in islet biology, it is likely that we will see more studies combining single-cell transcriptome and proteome data with epigenetic and spatially resolved technologies, getting us closer to a complete and comprehensive view of islet function in health and disease.

ACKNOWLEDGEMENTS

We apologize to our colleagues whose references were omitted owing to space constraints. This work was supported by National Institutes of Health grants DK068471 and DK078803 and an Iacocca Family Foundation fellowship to C.Z.

CONFLICT OF INTEREST

None declared.

REFERENCES

[1] Caicedo, A., 2013. Paracrine and autocrine interactions in the human islet: more than meets the eye. Seminars in Cell & Developmental Biology 24(1): 11–21.

[2] Di Cairano, E.S., Moretti, S., Marciani, P., Sacchi, V.F., Castagna, M., Davalli, A., et al., 2016. Neurotransmitters and neuropeptides: new players in the control of islet of Langerhans' cell mass and function. Journal of Cell Physiology 231(4):756–767.

[3] Roscioni, S.S., Migliorini, A., Gegg, M., Lickert, H., 2016. Impact of islet architecture on beta-cell heterogeneity, plasticity and function. Nature Reviews. Endocrinology 12(12):695–709.

[4] Salomon, D., Meda, P., 1986. Heterogeneity and contact-dependent regulation of hormone secretion by individual B cells. Experimental Cell Research 162(2):507–520.

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

[5] Van Schravendijk, C.F., Kiekens, R., Pipeleers, D.G., 1992. Pancreatic beta cell heterogeneity in glucose-induced insulin secretion. Journal of Biological Chemistry 267(30):21344—21348.

[6] Giordano, E., Cirulli, V., Bosco, D., Rouiller, D., Halban, P., Meda, P., 1993. B-cell size influences glucose-stimulated insulin secretion. American Journal of Physiology 265(2 Pt 1):C358—C364.

[7] Stefan, Y., Meda, P., Neufeld, M., Orci, L., 1987. Stimulation of insulin secretion reveals heterogeneity of pancreatic B cells in vivo. Journal of Clinical Investigation 80(1):175—183.

[8] Giordano, E., Bosco, D., Cirulli, V., Meda, P., 1991. Repeated glucose stimulation reveals distinct and lasting secretion patterns of individual rat pancreatic B cells. Journal of Clinical Investigation 87(6):2178—2185.

[9] Pipeleers, D.G., 1992. Heterogeneity in pancreatic beta-cell population. Diabetes 41(7):777—781.

[10] Kiekens, R., In 't Veld, P., Mahler, T., Schuit, F., Van De Winkel, M., Pipeleers, D., 1992. Differences in glucose recognition by individual rat pancreatic B cells are associated with intercellular differences in glucose-induced biosynthetic activity. Journal of Clinical Investigation 89(1):117—125.

[11] Ling, Z., Chen, M.C., Smismans, A., Pavlovic, D., Schuit, F., Eizirik, D.L., et al., 1998. Intercellular differences in interleukin 1beta-induced suppression of insulin synthesis and stimulation of noninsulin protein synthesis by rat pancreatic beta-cells. Endocrinology 139(4):1540—1545.

[12] Van De Winkel, M., Pipeleers, D., 1983. Autofluorescence-activated cell sorting of pancreatic islet cells: purification of insulin-containing B-cells according to glucose-induced changes in cellular redox state. Biochemical and Biophysical Research Communications 114(2):835—842.

[13] Pipeleers, D., 1987. The biosociology of pancreatic B cells. Diabetologia 30(5):277—291.

[14] Rui, J., Deng, S., Arazi, A., Perdigoto, A.L., Liu, Z., Herold, K.C., 2017. β cells that resist immunological attack develop during progression of autoimmune diabetes in NOD mice. Cell Metabolism.

[15] Grapengiesser, E., Gylfe, E., Hellman, B., 1988. Glucose-induced oscillations of cytoplasmic Ca2+ in the pancreatic beta-cell. Biochemical and Biophysical Research Communications 151(3):1299—1304.

[16] Asada, N., Shibuya, I., Iwanaga, T., Niwa, K., Kanno, T., 1998. Identification of alpha- and beta-cells in intact isolated islets of Langerhans by their characteristic cytoplasmic Ca2+ concentration dynamics and immunocytochemical staining. Diabetes 47(5):751—757.

[17] Nadal, A., Quesada, I., Soria, B., 1999. Homologous and heterologous asynchronicity between identified alpha-, beta- and delta-cells within intact islets of Langerhans in the mouse. Journal of Physiology 517(Pt 1):85—93.

[18] Herchuelz, A., Pochet, R., Pastiels, C., Van Praet, A., 1991. Heterogeneous changes in [Ca2+]i induced by glucose, tolbutamide and K+ in single rat pancreatic B cells. Cell Calcium 12(8):577—586.

[19] Hodson, D.J., Mitchell, R.K., Bellomo, E.A., Sun, G., Vinet, L., Meda, P., et al., 2013. Lipotoxicity disrupts incretin-regulated human beta cell connectivity. Journal of Clinical Investigation 123(10):4182—4194.

[20] Stozer, A., Gosak, M., Dolensek, J., Perc, M., Marhl, M., Rupnik, M.S., et al., 2013. Functional connectivity in islets of Langerhans from mouse pancreas tissue slices. PLoS Computational Biology 9(2):e1002923.

[21] Johnston, N.R., Mitchell, R.K., Haythorne, E., Pessoa, M.P., Semplici, F., Ferrer, J., et al., 2016. Beta cell hubs dictate pancreatic islet responses to glucose. Cell Metabolism 24(3):389—401.

[22] Hellerström, C., Swenne, I., 1985. The diabetic pancreas. In: Volk, B.W., Arquilla, E.R. (Eds.). Springer. p. 53—79.

[23] Bader, E., Migliorini, A., Gegg, M., Moruzzi, N., Gerdes, J., Roscioni, S.S., et al., 2016. Identification of proliferative and mature beta-cells in the islets of Langerhans. Nature 535(7612):430—434.

[24] Krishnamurthy, J., Ramsey, M.R., Ligon, K.L., Torrice, C., Koh, A., Bonner-Weir, S., et al., 2006. p16INK4a induces an age-dependent decline in islet regenerative potential. Nature 443(7110):453—457.

[25] Helman, A., Klochendler, A., Azazmeh, N., Gabai, Y., Horwitz, E., Anzi, S., et al., 2016. p16(Ink4a)-induced senescence of pancreatic beta cells enhances insulin secretion. Nature Medicine 22(4):412—420.

[26] Benner, C., van der Meulen, T., Caceres, E., Tigyi, K., Donaldson, C.J., Huising, M.O., 2014. The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. BMC Genomics 15:620.

[27] DiGruccio, M.R., Mawla, A.M., Donaldson, C.J., Noguchi, G.M., Vaughan, J., Cowing-Zitron, C., et al., 2016. Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets. Molecular Metabolism 5(7):449—458.

[28] Avrahami, D., Li, C., Zhang, J., Schug, J., Avrahami, R., Rao, S., et al., 2015. Aging-dependent demethylation of regulatory elements correlates with chromatin state and improved beta cell function. Cell Metabolism 22(4):619—632.

[29] Ku, G.M., Kim, H., Vaughn, I.W., Hangauer, M.J., Myung Oh, C., German, M.S., et al., 2012. Research resource: RNA-Seq reveals unique features of the pancreatic beta-cell transcriptome. Molecular Endocrinology 26(10):1783—1792.

[30] Bramswig, N.C., Everett, L.J., Schug, J., Dorrell, C., Liu, C., Luo, Y., et al., 2013. Epigenomic plasticity enables human pancreatic alpha to beta cell reprogramming. Journal of Clinical Investigation 123(3):1275—1284.

[31] Dorrell, C., Schug, J., Canaday, P.S., Russ, H.A., Tarlow, B.D., Grompe, M.T., et al., 2016. Human islets contain four distinct subtypes of beta cells. Nature Communications 7:11756.

[32] Nica, A.C., Ongen, H., Irminger, J.C., Bosco, D., Berney, T., Antonarakis, S.E., et al., 2013. Cell-type, allelic, and genetic signatures in the human pancreatic beta cell transcriptome. Genome Research 23(9):1554—1562.

[33] Hrvatin, S., Deng, F., O'Donnell, C.W., Gifford, D.K., Melton, D.A., 2014. MARIS: method for analyzing RNA following intracellular sorting. PLoS One 9(3):e89459.

[34] Hald, J., Galbo, T., Rescan, C., Radzikowski, L., Sprinkel, A.E., Heimberg, H., et al., 2012. Pancreatic islet and progenitor cell surface markers with cell sorting potential. Diabetologia 55(1):154—165.

[35] Pechhold, S., Stouffer, M., Walker, G., Martel, R., Seligmann, B., Hang, Y., et al., 2009. Transcriptional analysis of intracytoplasmically stained, FACS-purified cells by high-throughput, quantitative nuclease protection. Nature Biotechnology 27(11):1038—1042.

[36] Dorrell, C., Schug, J., Lin, C.F., Canaday, P.S., Fox, A.J., Smirnova, O., et al., 2011. Transcriptomes of the major human pancreatic cell types. Diabetologia 54(11):2832—2844.

[37] Gawad, C., Koh, W., Quake, S.R., 2016. Single-cell genome sequencing: current state of the science. Nature Reviews Genetics 17(3):175—188.

[38] Liu, S., Trapnell, C., 2016. Single-cell transcriptome sequencing: recent advances and remaining challenges. F1000Res 5.

[39] Su, Y., Shi, Q., Wei, W., 2017. Single cell proteomics in biomedicine: high-dimensional data acquisition, visualization and analysis. Proteomics.

[40] Wang, Y.J., Golson, M.L., Schug, J., Traum, D., Liu, C., Vivek, K., et al., 2016. Single-cell mass cytometry analysis of the human endocrine pancreas. Cell Metabolism 24(4):616—626.

[41] Li, J., Klughammer, J., Farlik, M., Penz, T., Spittler, A., Barbieux, C., et al., 2016. Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. EMBO Reports 17(2):178—187.

[42] Muraro, M.J., Dharmadhikari, G., Grun, D., Groen, N., Dielen, T., Jansen, E., et al., 2016. A single-cell transcriptome atlas of the human pancreas. Cell Systems 3(4), 385—394 e383.

[43] Segerstolpe, A., Palasantza, A., Eliasson, P., Andersson, E.M., Andreasson, A.C., Sun, X., et al., 2016. Single-cell transcriptome profiling of human pancreatic islets in health and type 2 diabetes. Cell Metabolism 24(4):593—607.

[44] Zeng, C., Mulas, F., Sui, Y., Guan, T., Miller, N., Tan, Y., et al., 2017. Pseudotemporal ordering of single cells reveals metabolic control of postnatal beta-cell proliferation. Cell Metabolism 25(5):1160—1175.

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

[45] Baron, M., Veres, A., Wolock, S.L., Faust, A.L., Gaujoux, R., Vetere, A., et al., 2016. A single-cell transcriptomic map of the human and mouse pancreas reveals inter- and intra-cell population structure. Cell Systems 3(4), 346–360 e344.

[46] Xin, Y., Okamoto, H., Kim, J., Ni, M., Adler, C., Cavino, K., et al., 2016. Single-cell RNAseq reveals that pancreatic beta-cells from very old male mice have a young gene signature. Endocrinology 157(9):3431–3438.

[47] Lawlor, N., George, J., Bolisetty, M., Kursawe, R., Sun, L., Sivakamasundari, V., et al., 2017. Single-cell transcriptomes identify human islet cell signatures and reveal cell-type-specific expression changes in type 2 diabetes. Genome Research 27(2):208–222.

[48] Xin, Y., Kim, J., Okamoto, H., Ni, M., Wei, Y., Adler, C., et al., 2016. RNA sequencing of single human islet cells reveals type 2 diabetes genes. Cell Metabolism 24(4):608–615.

[49] Xin, Y., Kim, J., Ni, M., Wei, Y., Okamoto, H., Lee, J., et al., 2016. Use of the Fluidigm C1 platform for RNA sequencing of single mouse pancreatic islet cells. Proceedings of the National Academy of Sciences of the United States of America 113(12):3293–3298.

[50] Jansson, E.T., Comi, T.J., Rubakhin, S.S., Sweedler, J.V., 2016. Single cell peptide heterogeneity of rat islets of Langerhans. ACS Chemical Biology 11(9):2588–2595.

[51] Bonner-Weir, S., Aguayo-Mazzucato, C., 2016. Physiology: pancreatic beta-cell heterogeneity revisited. Nature 535(7612):365–366.

[52] Yang, Q., Graham, T.E., Mody, N., Preitner, F., Peroni, O.D., Zabolotny, J.M., et al., 2005. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436(7049):356–362.

[53] Taneera, J., Lang, S., Sharma, A., Fadista, J., Zhou, Y., Ahlqvist, E., et al., 2012. A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. Cell Metabolism 16(1):122–134.

[54] Akerfeldt, M.C., Laybutt, D.R., 2011. Inhibition of Id1 augments insulin secretion and protects against high-fat diet-induced glucose intolerance. Diabetes 60(10):2506–2514.

[55] Zhou, P., Robles-Murguia, M., Mathew, D., Duffield, G.E., 2016. Impaired thermogenesis and a molecular signature for brown adipose tissue in Id2 null mice. Journal of Diabetes Research 2016:6785948.

[56] Satyanarayana, A., Klarmann, K.D., Gavrilova, O., Keller, J.R., 2012. Ablation of the transcriptional regulator Id1 enhances energy expenditure, increases insulin sensitivity, and protects against age and diet induced insulin resistance, and hepatosteatosis. FASEB Journal 26(1):309–323.

[57] Cutchins, A., Harmon, D.B., Kirby, J.L., Doran, A.C., Oldham, S.N., Skaflen, M., et al., 2012. Inhibitor of differentiation-3 mediates high fat diet-induced visceral fat expansion. Arteriosclerosis Thrombosis and Vascular Biology 32(2):317–324.

[58] Meier, J.J., Butler, A.E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., et al., 2008. Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 57(6):1584–1594.

[59] Teta, M., Long, S.Y., Wartschow, L.M., Rankin, M.M., Kushner, J.A., 2005. Very slow turnover of beta-cells in aged adult mice. Diabetes 54(9):2557–2567.

[60] Spitzer, M.H., Nolan, G.P., 2016. Mass cytometry: single cells, many features. Cell 165(4):780–791.

[61] Wang, Y.J., Schug, J., Won, K.J., Liu, C., Naji, A., Avrahami, D., et al., 2016. Single-cell transcriptomics of the human endocrine pancreas. Diabetes 65(10):3028–3038.

[62] Wang, P., Alvarez-Perez, J.C., Felsenfeld, D.P., Liu, H., Sivendran, S., Bender, A., et al., 2015. A high-throughput chemical screen reveals that harmine-mediated inhibition of DYRK1A increases human pancreatic beta cell replication. Nature Medicine 21(4):383–388.

[63] Shen, W., Taylor, B., Jin, Q., Nguyen-Tran, V., Meeusen, S., Zhang, Y.Q., et al., 2015. Inhibition of DYRK1A and GSK3B induces human beta-cell proliferation. Nature Communications 6:8372.

[64] Wei, R., Hong, T., 2016. Lineage reprogramming: a promising road for pancreatic beta cell regeneration. Trends in Endocrinology and Metabolism 27(3):163–176.

[65] Thorel, F., Nepote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S., et al., 2010. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 464(7292):1149–1154.

[66] Li, J., Casteels, T., Frogne, T., Ingvorsen, C., Honore, C., Courtney, M., et al., 2017. Artemisinins target GABAA receptor signaling and impair alpha cell identity. Cell 168(1–2), 86–100 e115.

[67] Ben-Othman, N., Vieira, A., Courtney, M., Record, F., Gjernes, E., Avolio, F., et al., 2017. Long-term GABA administration induces alpha cell-mediated beta-like cell neogenesis. Cell 168(1–2), 73–85 e11.

[68] Klochendler, A., Caspi, I., Corem, N., Moran, M., Friedlich, O., Elgavish, S., et al., 2016. The genetic program of pancreatic beta-cell replication in vivo. Diabetes 65(7):2081–2093.

[69] Leiter, E.H., Premdas, F., Harrison, D.E., Lipson, L.G., 1988. Aging and glucose homeostasis in C57BL/6J male mice. FASEB Journal 2(12):2807–2811.

[70] Jermendy, A., Toschi, E., Aye, T., Koh, A., Aguayo-Mazzucato, C., Sharma, A., et al., 2011. Rat neonatal beta cells lack the specialised metabolic phenotype of mature beta cells. Diabetologia 54(3):594–604.

[71] Sharma, R.B., O'Donnell, A.C., Stamateris, R.E., Ha, B., McCloskey, K.M., Reynolds, P.R., et al., 2015. Insulin demand regulates beta cell number via the unfolded protein response. Journal of Clinical Investigation 125(10):3831–3846.

[72] Asplund, K., Westman, S., Hellerstrom, C., 1969. Glucose stimulation of insulin secretion from the isolated pancreas of foetal and newborn rats. Diabetologia 5(4):260–262.

[73] Lavine, R.L., Chick, W.L., Like, A.A., Makdisi, T.W., 1971. Glucose tolerance and insulin secretion in neonatal and adult mice. Diabetes 20(3):134–139.

[74] van de Bunt, M., Manning Fox, J.E., Dai, X., Barrett, A., Grey, C., Li, L., et al., 2015. Transcript expression data from human islets links regulatory signals from genome-wide association studies for type 2 diabetes and glycemic traits to their downstream effectors. PLoS Genetics 11(12):e1005694.

[75] Fadista, J., Vikman, P., Laakso, E.O., Mollet, I.G., Esguerra, J.L., Taneera, J., et al., 2014. Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. Proceedings of the National Academy of Sciences of the United States of America 111(38): 13924–13929.

[76] Ichii, H., Inverardi, L., Pileggi, A., Molano, R.D., Cabrera, O., Caicedo, A., et al., 2005. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. American Journal of Transplantation 5(7):1635–1645.

[77] Kloppel, G., Lohr, M., Habich, K., Oberholzer, M., Heitz, P.U., 1985. Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. Survey and Synthesis of Pathology Research 4(2):110–125.

[78] Yoon, K.H., Ko, S.H., Cho, J.H., Lee, J.M., Ahn, Y.B., Song, K.H., et al., 2003. Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. Journal of Clinical Endocrinology and Metabolism 88(5):2300–2308.

[79] Butler, A.E., Janson, J., Soeller, W.C., Butler, P.C., 2003. Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. Diabetes 52(9):2304–2314.

[80] Flamez, D., Roland, I., Berton, A., Kutlu, B., Dufrane, D., Beckers, M.C., et al., 2010. A genomic-based approach identifies FXYD domain containing ion transport regulator 2 (FXYD2)gammaa as a pancreatic beta cell-specific biomarker. Diabetologia 53(7):1372–1383.

[81] Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., et al., 1999. Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. Science 283(5404):981–985.

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

[82] Seron, K., Couturier, C., Belouzard, S., Bacart, J., Monte, D., Corset, L., et al., 2011. Endospanins regulate a postinternalization step of the leptin receptor endocytic pathway. Journal of Biological Chemistry 286(20):17968—17981.

[83] Talchai, C., Xuan, S., Lin, H.V., Sussel, L., Accili, D., 2012. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 150(6): 1223—1234.

[84] Wang, Z., York, N.W., Nichols, C.G., Remedi, M.S., 2014. Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. Cell Metabolism 19(5):872—882.

[85] Cinti, F., Bouchi, R., Kim-Muller, J.Y., Ohmura, Y., Sandoval, P.R., Masini, M., et al., 2016. Evidence of beta-cell dedifferentiation in human type 2 diabetes. Journal of Clinical Endocrinology and Metabolism 101(3):1044—1054.

[86] Brennecke, P., Anders, S., Kim, J.K., Kolodziejczyk, A.A., Zhang, X., Proserpio, V., et al., 2013. Accounting for technical noise in single-cell RNA-seq experiments. Nature Methods 10(11):1093—1095.

[87] Risso, D., Ngai, J., Speed, T.P., Dudoit, S., 2014. Normalization of RNA-seq data using factor analysis of control genes or samples. Nature Biotechnology 32(9):896—902.

[88] Leek, J.T., 2014. svaseq: removing batch effects and other unwanted noise from sequencing data. Nucleic Acids Research 42(21).

[89] Stegle, O., Teichmann, S.A., Marioni, J.C., 2015. Computational and analytical challenges in single-cell transcriptomics. Nature Reviews Genetics 16(3):133—145.

[90] Shin, J., Berg, D.A., Zhu, Y., Shin, J.Y., Song, J., Bonaguidi, M.A., et al., 2015. Single-cell RNA-seq with waterfall reveals molecular cascades underlying adult neurogenesis. Cell Stem Cell 17(3):360—372.

[91] Buettner, F., Natarajan, K.N., Casale, F.P., Proserpio, V., Scialdone, A., Theis, F.J., et al., 2015. Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. Nature Biotechnology 33(2):155—160.

[92] Johnson, W.E., Li, C., Rabinovic, A., 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8(1): 118—127.

[93] Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., et al., 2014. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nature Biotechnology 32(4):381—386.

[94] Ji, Z., Ji, H., 2016. TSCAN: pseudo-time reconstruction and evaluation in single-cell RNA-seq analysis. Nucleic Acids Research 44(13):e117.

[95] Amir el, A.D., Davis, K.L., Tadmor, M.D., Simonds, E.F., Levine, J.H., Bendall, S.C., et al., 2013. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nature Biotechnology 31(6):545—552.

[96] Bendall, S.C., Davis, K.L., Amir el, A.D., Tadmor, M.D., Simonds, E.F., Chen, T.J., et al., 2014. Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. Cell 157(3):714—725.

[97] Krishnaswamy, S., Spitzer, M.H., Mingueneau, M., Bendall, S.C., Litvin, O., Stone, E., et al., 2014. Systems biology. Conditional density-based analysis of T cell signaling in single-cell data. Science 346(6213):1250689.

[98] Newell, E.W., Cheng, Y., 2016. Mass cytometry: blessed with the curse of dimensionality. Nature Immunology 17(8):890—895.

[99] Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., Tyagi, S., 2008. Imaging individual mRNA molecules using multiple singly labeled probes. Nature Methods 5(10):877—879.

[100] Lyubimova, A., Itzkovitz, S., Junker, J.P., Fan, Z.P., Wu, X., van Oudenaarden, A., 2013. Single-molecule mRNA detection and counting in mammalian tissue. Nature Protocols 8(9):1743—1758.

[101] Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B.P., Landegren, U., 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 265(5181):2085—2088.

[102] Lizardi, P.M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D.C., Ward, D.C., 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nature Genetics 19(3):225—232.

[103] Kern, D., Collins, M., Fultz, T., Detmer, J., Hamren, S., Peterkin, J.J., et al., 1996. An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. Journal of Clinical Microbiology 34(12):3196—3202.

[104] Battich, N., Stoeger, T., Pelkmans, L., 2013. Image-based transcriptomics in thousands of single human cells at single-molecule resolution. Nature Methods 10(11):1127—1133.

[105] Crosetto, N., Bienko, M., van Oudenaarden, A., 2015. Spatially resolved transcriptomics and beyond. Nature Reviews Genetics 16(1):57—66.

[106] Lovatt, D., Ruble, B.K., Lee, J., Dueck, H., Kim, T.K., Fisher, S., et al., 2014. Transcriptome in vivo analysis (TIVA) of spatially defined single cells in live tissue. Nature Methods 11(2):190—196.

[107] Giesen, C., Wang, H.A., Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B., et al., 2014. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nature Methods 11(4):417—422.

[108] Pettit, J.B., Tomer, R., Achim, K., Richardson, S., Azizi, L., Marioni, J., 2014. Identifying cell types from spatially referenced single-cell expression datasets. PLoS Computational Biology 10(9):e1003824.

[109] Stergachis, A.B., Neph, S., Reynolds, A., Humbert, R., Miller, B., Paige, S.L., et al., 2013. Developmental fate and cellular maturity encoded in human regulatory DNA landscapes. Cell 154(4):888—903.

[110] Wang, A., Yue, F., Li, Y., Xie, R., Harper, T., Patel, N.A., et al., 2015. Epigenetic priming of enhancers predicts developmental competence of hESC-derived endodermal lineage intermediates. Cell Stem Cell 16(4):386—399.

[111] Consortium, E.P., 2012. An integrated encyclopedia of DNA elements in the human genome. Nature 489(7414):57—74.

[112] Rotem, A., Ram, O., Shoresh, N., Sperling, R.A., Goren, A., Weitz, D.A., et al., 2015. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. Nature Biotechnology 33(11):1165—1172.

[113] Nagano, T., Lubling, Y., Stevens, T.J., Schoenfelder, S., Yaffe, E., Dean, W., et al., 2013. Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature 502(7469):59—64.

[114] Buenrostro, J.D., Wu, B., Litzenburger, U.M., Ruff, D., Gonzales, M.L., Snyder, M.P., et al., 2015. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523(7561):486—490.

[115] Nagano, T., Lubling, Y., Yaffe, E., Wingett, S.W., Dean, W., Tanay, A., et al., 2015. Single-cell Hi-C for genome-wide detection of chromatin interactions that occur simultaneously in a single cell. Nature Protocols 10(12):1986—2003.

[116] Dey, S.S., Kester, L., Spanjaard, B., Bienko, M., van Oudenaarden, A., 2015. Integrated genome and transcriptome sequencing of the same cell. Nature Biotechnology 33(3):285—289.

[117] Macaulay, I.C., Haerty, W., Kumar, P., Li, Y.I., Hu, T.X., Teng, M.J., et al., 2015. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. Nature Methods 12(6):519—522.

 © 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).