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Is a β cell a β cell?

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Abstract

Purpose of review—This report examines recent publications identifying phenotypic and functional heterogeneity among pancreatic β cells and investigating their potential roles in normal and abnormal islet function. The development of new methods and tools for the study of individual islet cells has produced a surge of interest in this topic.

Recent findings—Studies of β cell maturation and pregnancy-induced proliferation have identified changes in serotonin and transcription factors SIX2/3 expression as markers of temporal heterogeneity. Structural and functional heterogeneity in the form of functionally distinct 'hub' and 'follower' β cells was found in mouse islets. Heterogeneous expression of Fltp (in mouse β cells) and ST8SIA1 and CD9 (in human β cells) were associated with distinct functional potential. Several impressive reports describing the transcriptomes of individual β cells were also published in recent months. Some of these reveal previously unknown β cell subpopulations.

Summary—A wealth of information on functional and phenotypic heterogeneity has been collected recently, including the transcriptomes of individual β cells and the identities of functionally distinct β cell subpopulations. Several studies suggest the existence of two broad categories: a more proliferative but less functional and a less proliferative but more functional β cell type. The identification of functionally distinct subpopulations and their association with type 2 diabetes underlines the potential clinical importance of these investigations.

Keywords

embryonic stem/induced pluripotent stem; glucose-stimulated insulin secretion; single-cell transcriptome; type 1 diabetes; type 2 diabetes

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INTRODUCTION

Insulin secretion by pancreatic β cells is the central endocrine regulator of carbohydrate metabolism. Because the presence of insulin is the defining characteristic of these cells, by definition, all β cells express insulin. However, many types of functional (e.g. insulin release kinetics [1]), expression phenotype (e.g. PSA-NCAM distribution [2]), and structural phenotype (e.g. cell size [3]) heterogeneity have been observed in humans and rodents over decades of study. Although these cells have been subject to unusual scrutiny because of their critical association with diabetes, the apparent extent of heterogeneity is substantial. Understanding this characteristic and how it contributes to β cell function will be important for guided ES/iPS cell differentiation and adult cell reprogramming protocols intended to generate replacement β cells for type 1 diabetes (T1D). Heterogeneity also seems to impact the behavior of β cells during type 1 diabetes (T2D) and may therefore be involved in progression of the disease.

In this review, we discuss recent studies relevant to normal, pathological, and induced β cell heterogeneity (Note: for a comprehensive survey of the topic including historical work, we recommend a recent review by Roscioni *et al.* [4]). Thanks in part to new methods for single-cell analysis and the identification of new antibodies and genetic markers of β cell heterogeneity, interest in this topic has recently increased. Although many challenges remain, the field seems poised for rapid progress.

RECENT DISCOVERIES OF β-CELL HETEROGENEITY IN NORMAL CONDITIONS

Although most β cell development occurs *in utero*, studies of temporal heterogeneity in the form of postnatal β cell maturation have yielded important insights into β cell identity. A report by Stolovich-Rain *et al.* examined mouse β cells preweaning and postweaning and found that the more ‘mature’ postweaning cells had a higher proliferative/regenerative capacity [5]. Efforts to regenerate mature β cells by inducing a transiently-primitive state should therefore consider that younger β cells are not necessarily progenitor-like.

The enhanced metabolic requirements of β cells during pregnancy have been shown to induce temporal heterogeneity [6,7]. When murine islets of either sex were transplanted to a female mouse, serotonin expression was shown to be induced in a subset of β cells. The authors speculate that the serotonin-expressing subset may correspond to the pregnancy-induced proliferative (or nonproliferative) β cell subpopulation, but this is not yet clear.

Recently, a comprehensive examination of age-determined gene expression in FACS-isolated human endocrine and exocrine pancreatic cells was reported [8]. Among the many interesting observations was the identification of differential expression of transcription factors SIX2 and SIX3 during postnatal β cell maturation. This report showed that SIX2/3 expression was absent in juvenile (<9 yo) human β cells but abundant in adult (>28 yo) β cells. The level of SIX3 expression was associated with β cell function; lentiviral introduction of SIX3 expression to juvenile human islets caused a conversion to an adult-like state.

Heterogeneity in the interaction and arrangement of β cells within the islet is also an area of recent investigation. Johnston *et al.* [9▪▪] monitored of calcium flux in intact mouse islets and identified rare 'hub' β cells which were consistently the first with electrical activity and the last to cease. These appeared to act as pacemakers for abundant 'follower' β cells via gap junction signaling. Using an InsCre/floxed eNpHR3.0-EYFP mouse, the effects of laser silencing of individual β cells on intact islet function were then monitored. Silencing of a single hub cell perturbed calcium dynamics and insulin secretion (monitored indirectly by zinc release), whereas targeting a follower cell had little effect. Hub cells were found to be randomly located throughout the islet, and had reduced levels of insulin and maturity-associated β cell transcription factors. Glucokinase levels were increased, however, and the authors speculate that hypersensitivity to glucose may explain their ability to respond more rapidly than other cells. In another report examining human cells, functional heterogeneity was assessed by statistical clustering of cellular Ca^{2+} responses to glucose stimulation, revealing two populations of juvenile human β cells distinguished by Ca^{2+} flux oscillation frequency and magnitude [10]. The authors speculate that these populations may correspond to mature and immature β cell types.

HETEROGENEITY IN HEALTHY AND DIABETIC ENVIRONMENTS: PHENOTYPE AND FUNCTION

Observations of differential responses among large populations of β cells simultaneously exposed to glucose (e.g. Kiekens *et al.* [11]) has suggested that β cells exhibit differences in the amount or distribution of proteins involved in glucose sensing and/or insulin secretion. A recent report which supports this idea describes heterogeneity in the level of glucose transporter Glut2 in mouse β cells [12]. Under de-differentiating conditions, a small proportion of β cells was observed with reduced Glut2 and a gene expression profile associated with decreased function. Because Glut2 is located on the cell surface, it should be possible to isolate live $Glut2^{lo}$ β cells and assess their function directly in future studies.

A recent report by Bader *et al.* [13▪▪] elegantly combines phenotypic heterogeneity and functional correlation with a structural model. Fltp, a gene associated with epithelial layer polarization, was observed in a large subset (80%) of mouse β cells. $Fltp^{+}$ cells were postmitotic and expressed genes associated with mature β cell function, whereas the $Fltp^{-}$ population had a higher frequency of proliferative cells during postnatal development and pregnancy. Although Fltp knockout mice showed only a minor deficit in insulin secretion, the apparent structural organization of $Fltp^{+}$ β cells into clusters in the islet core and $Fltp^{-}$ cells toward the periphery offers an intriguing potential connection heterogeneous gene expression, function, and physical organization of β cells.

A report of proliferative heterogeneity among human β cells was provided by Wang *et al.* [14▪]. In this first ever study of human endocrine cells by $CyTOF^2$ mass cytometry, islet cells were gated into hormone-defined endocrine populations and examined for expression of 18 additional proteins. Heterogeneity was observed among several of these markers including CD9 and ST8SIA1, and a small subpopulation of $Ki67^{+}$ cells was identified. This

proliferative population was not detected in analyses of T2D β cells, implying a diabetes-associated regenerative defect.

In a related study, we observed heterogeneous expression of CD9 and ST8SIA1 in human β cells [15▪▪]. These studies were not performed with the a-priori expectation that these proteins were regulators of human β cell function. ST8SIA1 was identified as the antigen bound by HIC0-3C5, a monoclonal antibody generated in a whole islet immunization screen and observed to label a subset of islet cells. CD9 mRNA was detected in FACS isolated β cells and was found to label a different, partially overlapping cell subset. When the four separable β cell populations (termed β1–β4) were isolated, the minor (β2–β4) populations had reduced GSIS. Although gene expression was broadly similar in the subtypes, significantly higher expression of transcription factor SIX3 and cation channel HCN1 were found in ST8SIA1^{+} (β2/β4) cells. No obvious patterns indicating differential proliferative capacity were observed, but genes and pathways associated with insulin secretion and mature β cell function were significantly enriched in ST8SIA1^{-} (β1/β3) cells. Inhibition of HCN channel activity in cultured islets with ivabradine increased basal insulin secretion and reduced GSIS, suggesting that differential activity of such channels might contribute to the observed differences in β cell subset function. Finally, the frequencies of the β cell subpopulations were found to be significantly different in islets from patients with T2D, although considerable donor-to-donor variation was observed, the frequency of β2 to β4 cells was significantly higher.

HETEROGENEITY IN HEALTHY AND DIABETIC ENVIRONMENTS: SINGLE-CELL TRANSCRIPTOME ANALYSES

Following the technical advancements of next-generation sequencing, transcriptomic profiling has broadened and deepened our understanding of biological systems and diseases [16–19]. A few years ago, single-cell transcriptome analysis became possible. This new tool has high potential to reveal the rare but key players which might be missed in the averaged transcriptome analysis.

Single-cell transcriptome data of human islets first appeared in the literature in 2016 [20▪]. Dissociated human islets were FACS-sorted into 96-well plates and single-cell transcriptomes obtained by Smart-Seq2; 64 of 70 cells passed sequencing quality control and grouped into seven clusters by principal component analysis (PCA). The Van oudenaarden group developed an automated platform using FACS, liquid handling robotics, and the CEL-Seq2 protocol. By a combination of StemID analysis [21] and manual annotation, nine islet populations were found and β cell subpopulations were revealed by use of a RaceID algorithm [22▪]. Comparing single-cell transcriptomes between healthy and T2D donors has been done by the Sandberg group [23▪]. FACS was used to dispense dissociated islet cells into 384-well plates for processing using Smart-Seq2. Ductal, acinar, and four hormone-producing cell populations were found, including five sub-populations within the β cell cluster. Another automated system, the Fluidigm C1 platform, used by the Gromada group [24▪–26▪]. Marker gene expression defined five mouse islet cell types, but no subpopulation of β cells was observed. A large percentage of captured cells were not

usable for analysis as contamination and damaging of the cell integrity may have occurred during the cell capture process. Surprisingly, only 151 genes were enriched in β cells, and young and old mice showed little change in the transcriptome profile. This method was also applied to human healthy and T2D islets, and they found 69 genes that were enriched in the β cell population, and 48 genes affected by T2D. Ingenuity pathway analysis (IPA) indicated that EIF2 signaling, oxidative phosphorylation, and mitochondrial dysfunction were altered in T2D donors. In T2D cells, *FXYD2* was found to be upregulated, whereas this gene was reported to be downregulated by the Sand-berg group [23▪]. The Kaestner group has also used the Fluidigm C1 platform to study more types of human samples, including healthy adults and children, and T1D and T2D adults. This group classified 430 islet cells into six cell types based on established marker genes. Their data indicated that healthy adults had very clear separation between different cell types, whereas children and T2D adults had much less distinct gene signatures between different cell types [27▪].

Another high-throughput droplet-microfluidic approach, termed InDrop, was recently developed [28]. This approach allows capture of thousands of individual cells in a short time along with a set of uniquely barcoded primers and has high capture efficiency and low noise profile. Using InDrop the Yanai group captured 12 000 islet cells from four healthy adults and two mouse strains, and assigned ~88% of cells to 13 and 14 cell clusters for mouse and human cells, respectively [29▪]. In addition, the expected cell populations, rare cell types such as ε and immune cells were detected. Furthermore, PCA detected two subpopulations which had variable gene expressions relevant to β cell function and the endoplasmic reticulum stress-inducible genes.

As shown by these studies, enormous challenges are intrinsically associated with human islet projects, even more so with T1D research. The Kaestner group made an attempt to study the single-cell transcriptome of adult T1D islets, but were only able to collect six β cells because of their scarcity [27▪]. To elucidate mechanisms underlying initiation and progression of this disease, our laboratory has worked extensively with the BBDR rat model, an autoimmune diabetes model. BBDR rats develop autoimmune diabetes after infection with Kilham Rat Virus, but remain diabetes-free in the absence of viral infection [30–34]. We also utilized the InDrop method to interrogate the single-cell transcriptomes of healthy adult BBDR rat islets. Because of several unique computational challenges associated with single-cell RNA-seq, such as multiplexing, low input, and a strong 3′-end bias, an end sequence analysis toolkit (ESAT) was developed that uses an extra UTR extension step to retrieve useful sequence reads (24%), which otherwise would be lost using traditional RNA-seq analysis methods [35]. With this protocol 93% captured cells and 8264 genes, compared to ~6000 genes from other studies, passed our quality control criteria. Independent component analysis revealed nine separate populations, with three distinct subpopulations of β cells. We also found two clusters with genes related to vascular development and one cluster enriched in immune function genes. We are currently using this improved method to study heterogeneity of islets, especially β cells, during the development of autoimmune diabetes. Dissecting heterogeneity of β cells is particularly important for detecting the initial pathological events of this disease. The BBDR model's strict kinetic development of diabetes and its close similarity with human T1D pathogenesis allows us to study β cells at early asymptomatic stages to provide new insights into this disease.

As shown above, various single-cell RNA-seq protocols are available. However, the general workflows are similar and involve islet dissociation, single-cell capture, cell lysis, mRNA capture and reverse transcription into cDNA, cDNA amplification, library construction and sequencing, and bioinformatics analyses. When we examined each step, the limitations came not only from bulk RNA-Seq, but also single-cell specific and islet/β cell biology-specific limitations. For example, once islets were dissociated, the cells' transcription profiles could have been altered. Cell capture methods, such as the Fluidigm C1 platform, could also introduce artifacts. Because one cell has only a very small amount of mRNA, cDNA needs to be amplified. Currently, the best capture rate is only 10% (such as InDrop), therefore the low abundance transcript information is likely not be included. In addition, hormone producing cells, especially β cells, have very high intrinsic levels of insulin transcripts, which allows even less capture power for low abundance transcripts. These limitations may be the reason for the variability of different studies published recently.

Both the single-cell transcriptome technologies in the wet lab and bioinformatics analysis tools are improving at a very fast pace. Meanwhile, we need to be alert for technical 'noise' when we use these methods. As biologists, we might improve data accuracy to extract biologically meaningful data through designing more appropriate experiments, or combining it with other technologies. For example, combining transcriptomics with techniques that provide spatial information, such as laser micro-dissection, will provide an additional layer of information [36]. Combining it with antibody staining will deliver protein-level information. Multiomics analyses from one cell will give us comprehensive information [37,38]. Finally, human samples are highly variable by nature, so developing better animal model systems to investigate human disease is crucial. Such models are the foundation for developing and validating new technologies, but we need to link animal models to humans to overcome species differences.

β-CELL HETEROGENEITY ASSOCIATED WITH INDUCED PLURIPOTENT STEM/EMBRYONIC STEM DIFFERENTIATION AND ADULT CELL REPROGRAMMING

The artificial generation of new β-like cells employs the conversion of other cell types, with varying degrees of efficiency and therefore varying degrees of 'β identity'. Ongoing studies will determine how closely such cells must resemble authentic β cells and the degree to which heterogeneity is tolerable or even desirable.

Significant improvements have been made in the in-vitro differentiation of human induced pluripotent stem/embryonic stem (iPS/ES) cells to a β-like fate. One goal of these studies is to avoid the production of polyhormonal cells which express other hormones in addition to insulin. Although heterogeneity in the form of β cell polyhormonality has been postulated as a feature of normal human islets, an emerging consensus suggests that such cells are exceptionally rare [23▪,25▪]. Thus, such cells are regarded as misprogrammed. Russ *et al.* investigated the origin of iPS/ES-derived polyhormonal cells and found that a modification of their protocol to prevent premature induction of NeuroG3 boosted the frequency of monohormonal β-like cells and dramatically reduced that of INS+GCG+ polyhormonal cells

[39]. Some of the off-target differentiation outcomes in iPS/ES procedures appear to be determined intrinsically by the cell source. A comparison of eight different human iPS and embryonic stem lines found that NKX6-1 induction frequencies ranged from 37 to 84% despite an identical differentiation protocol [40].

The reprogramming of adult cells to a β-like fate offers potential advantages over iPS/ES differentiation, such as potential use *in situ*. A recent report of in-situ reprogramming in mouse stomach and intestine found that subpopulations of enteroendocrine cells in these sites could initiate insulin expression upon targeted introduction of Pdx1, NeuroG3, and MafA [41]. Stomach cells were particularly well reprogrammed; when this approach was modified to employ transplantation of 'mini-organs' generated from reprogrammed stomach tissue, a significant reduction of hyperglycemia was achieved. Considerable heterogeneity of β-associated gene expression was observed among reprogrammed insulin$^+$ cells, suggesting potential for further refinement.

Consideration of the β cell subtypes present in healthy islets may be an important new consideration for iPS/ES differentiation and adult cell reprogramming methods. Monitoring the emergence (or absence) of these β cell subtypes may inform the improvement of differentiation and reprogramming methods.

CONCLUSION

The investigation of β cell heterogeneity is not new, but the development of new methods for determining its origins and consequences has yielded impressive recent progress. The specific nature of this heterogeneity is somewhat different in each report, but a general pattern of subpopulations distinguished by primitiveness, proliferative capacity, and the ability to perform typical β cell functions has emerged. Single-cell RNA-seq has proven to be a powerful tool to uncover expression heterogeneity and biological interactions. Pathway analyses should help to unify analyses using different methodology and species.

An understanding of the roles of β cell subtypes should help to guide efforts to produce replacement β cells. Quite logically, the focus has been to generate cells with the highest degree of glucose dependent insulin secretion. It may turn out that recreating the natural heterogeneity of these cells is equally important.

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KEY POINTS

- Recent studies have shown that human and mouse β cells exhibit many types of heterogeneity.
- Prospective identification of β cell subpopulations by cell surface-marking antibodies or transgenic mice containing tagged proteins has allowed the study of functionally distinct subtypes.
- Single-cell transcriptome studies have revealed β cell subpopulations distinguished by expression clustering, distinguishing characteristics include proliferation-associated genes.